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Abstract
Ten years after the first draft versions of the human genome were announced, technical progress in both DNA sequencing and ancient DNA analyses has allowed a research team around Ed Green and Svante Pa¨a¨bo to complete this task from infinitely more difficult hominid samples: a few pieces of bone originating from our closest, albeit extinct, relatives, the Neanderthals. Pulling the Neanderthal sequences out of a sea of contaminating environmental DNA impregnating the bones and at the same time avoiding the problems of contamination with modern human DNA is in itself a remarkable accomplishment. However, the crucial question in the long run is, what can we learn from such genomic data about hominid evolution?

Keywords
Neanderthal, Neanderthal Genome, Hominid Evolution

Invited Commentary

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Abstract Ten years after the first draft versions of the human genome were announced, technical progress in both DNA sequencing and ancient DNA analyses has allowed a research team around Ed Green and Svante Pääbo to complete this task from infinitely more difficult hominid samples: a few pieces of bone originating from our closest, albeit extinct, relatives, the Neanderthals. Pulling the Neanderthal sequences out of a sea of contaminating environmental DNA impregnating the bones and at the same time avoiding the problems of contamination with modern human DNA is in itself a remarkable accomplishment. However, the crucial question in the long run is, what can we learn from such genomic data about hominid evolution?

The first Neanderthal DNA sequences were reported in 1997 (Krings et al. 1997), which at the time, despite comprising a mere 370 bp of mitochondrial DNA (mtDNA), represented a major breakthrough in ancient DNA research. The sequences were not only the first DNA sequences reported for an extinct hominid form, they also suggested that, at least with regard to this genetic locus, modern humans and Neanderthals were quite distinct. Since then, mtDNA sequences ranging from 31 bp (Serre et al. 2004) to full mitochondrial genomes (Green et al. 2008, Briggs et al. 2009) from more than a dozen Neanderthal individuals have been published, allowing a range of conclusions about the relationship of modern humans and Neanderthals. For example, it could be shown that modern human and Neanderthal mtDNA sequences form two distinct groups in all phylogenetic analyses (e.g., Schmitz et al. 2002; Briggs et al. 2009). The data have also been used to draw conclusions about gene flow between Neanderthals and modern humans, although, depending on the demographic model used, the estimates for gene flow from Neanderthals into the early modern human gene pool ranged from up to 25% (Serre et al. 2004) to less than 0.1% (Currat and Excoffier 2004).

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As interesting as these earlier studies were, they were limited by the fact that mtDNA represents only a single, strictly maternally inherited locus. Moreover, modern human mtDNA sequences obtained from Neanderthal bones were generally (and most times probably correctly so) assumed to be derived from contamination with DNA from excavators, curators, or other researchers (Serre et al. 2004). Therefore, mtDNA sequences only allowed detecting gene flow from female Neanderthals into modern humans if it occurred to a sufficient extent that it would still be visible today and not lost by genetic drift (Nordborg 1998; Serre et al. 2004) or alternatively be detected in an ancient bone unambiguously assigned to a modern human individual (Serre et al. 2004). Fortunately, the introduction of hybridization capture methods such as primer extension capture (Briggs et al. 2009) now seems to allow judging the authenticity of ancient hominid sequences in the absence of knowledge about the taxonomic affiliation of a bone (Krause et al. 2010a). Thus, in the future it may become possible that modern human mtDNA sequences can be trusted even when obtained from a Neanderthal fossil.

However, the main problem of mtDNA lies in the fact that it represents a mere 0.0005% of a complete hominid genome and, moreover, as noted above is inherited strictly through the female lineage. Therefore, for a better understanding of the evolutionary relationship of modern humans and Neanderthals, analyzing a substantial part of the remaining 99.9995% of the genome encoded in the nucleus was required. For a long time, technical problems with the analysis of ancient DNA prevented substantial parts of the nuclear genome from being sequenced, but progress in ancient DNA analyses and especially DNA sequencing technology (Margulies et al. 2005; Poinar et al. 2006; Schuster 2008) from 2005 onward have made such an endeavor realistic. At the end of 2006, two publications reported the first nuclear DNA sequences from Neanderthals (Green et al. 2006; Noonan et al. 2006). One of the studies (Noonan et al. 2006) was based on directly cloning Neanderthal DNA extract into a bacterial library followed by sequencing thousands of clones, a method that had previously been used for sequencing about 27,000 bp of cave bear nuclear DNA (Noonan et al. 2005), whereas the second study (Green et al. 2006) used the then quite new 454 technology (Margulies et al. 2005), which had before been used for sequencing about 13 million bp of mammoth DNA (Poinar et al. 2006). In total, Noonan et al. obtained about 60,000 bp of Neanderthal DNA sequence, while Green et al. ended up with about 1 million bp. When comparing the amount of sequence data obtained, it became immediately clear that if a complete genome was to be obtained, the 454 technology adopted by Green et al. would be more suitable.

Despite the fact that both studies used aliquots of the same Neanderthal extract, they arrived at quite different conclusions with regard to biological questions such as the time of divergence or the amount of gene flow between modern humans and Neanderthals. Whereas Noonan et al. found no evidence for gene flow from modern humans into Neanderthals, Green et al. suggested that a substantial amount of gene flow had taken place. Green et al. also suggested a

divergence time of modern humans and Neanderthals that was not only substantially younger than that obtained by Noonan et al. but also at odds with almost all interpretations of the fossil record. Interestingly, both discrepancies are readily explained if contamination with modern human DNA affected the results by Green et al. It therefore came not as a big surprise that a re-analysis of both data sets concluded that the data from Green et al. were heavily affected by contamination, which may have comprised as much as 80% of the data set (Wall and Kim 2007). Although Green et al. argue that the contamination level in their original data set is lower than claimed by Wall and Kim, they concede that up to 40% of the data may consist of contaminating modern human DNA (Green et al. 2009, 2010). This result created somewhat of a paradox: the data obtained by Noonan et al. seemed reliable, but their methodology would not allow obtaining a substantial part of a Neanderthal genome with realistic effort and acceptable damage to the specimens. In contrast, while this aim seemed realistic using the methodology from Green et al., it was not clear whether it would be possible obtaining reliable data.

Gene Flow or No Gene Flow? Now that Green et al. have published a draft version of the Neanderthal genome, one of the first questions that arises is of the reliability of the data. In their analyses of the Neanderthal genome draft, Green et al. use several tests to investigate their data for potential contamination with modern human DNA using mitochondrial, y-chromosomal, and autosomal DNA sequences. Invariably of the test system applied, they arrive at a contamination level of less than 1%. These results seem to be solid and show that it is at least in some cases possible to study the nuclear genome of extinct hominids. Interestingly, when comparing the conclusions of the 2006 and the 2010 papers, the occurrence of signs of modern European human DNA in the data set is interpreted very differently. In 2006, it was taken as evidence for substantial gene flow from modern humans into the Neanderthal gene pool, whereas in 2010 any traces of modern human DNA are interpreted as signs of contamination. However, there are several good reasons for believing the later interpretation. First, all Neanderthal samples analyzed by Green et al. date to at least 38,000 ^{14}C years before present (BP), clearly predating the first direct evidence for anatomically modern humans in Europe, which comes from Pestera cu Oase, dating to 35,000 ^{14}C years BP (Trinkaus et al. 2003). Given these ages, it is not expected that any evidence of gene flow from modern humans into Neanderthals could be found in these samples. If ancient DNA should provide meaningful input on the discussion of gene flow between modern humans and Neanderthals in Europe, it will be necessary to analyze either some of the youngest Neanderthal samples or some of the oldest anatomically modern humans in Europe, such as the remains from Pestera cu Oase. However, as recently reviewed by Jöris and Street (2008), there is rather limited support for the claim that modern humans and Neanderthals overlapped temporally in Europe at all. Thus, it may not be entirely surprising that to date no convincing evidence for

gene flow between modern humans and Neanderthals in Europe has been found. Second, for two of the three genetic markers analyzed by Green et al., gene flow can be excluded as explanation for the occurrence of modern human sequences. The first of these markers is the mitochondrial genome. As it is inherited as a single nonrecombining genetic locus, any mtDNA sequences can be derived either from a Neanderthal or a modern human mitochondrial genome. Therefore, any fragments containing sequence motifs specific for modern humans must be derived from contamination, which is the case for less than 1% of the mtDNA sequence reads in the data set. Similarly, the overall data set convincingly shows that the sequenced Neanderthal samples are all from female individuals. Thus, any sequence convincingly assigned to the nonrecombining male-specific part of the Y chromosome must similarly be derived from modern human contamination. Again, this measure suggests a contamination level of less than 1%. The only DNA sequences that could indicate gene flow are autosomal sequences. However, the extent of modern human contribution in the data set is less than 1% for this class of genetic markers as well, in the same range as for mtDNA and Y-chromosomal sequences. The most parsimonious explanation for the appearance of autosomal modern human sequences in the data set lies, therefore, as for the other two genetic markers, in a small amount of contamination of the data with modern human DNA.

Interestingly, Green et al. do find evidence that gene flow between Neanderthals and modern humans took place, albeit much earlier, around 50,000 to 80,000 yrs ago. By comparing the Neanderthal sequences to the nuclear genomes of several modern humans, they find that traces of 1 to 4% Neanderthal contribution are found not only in the European but also in the Asian human gene pool, a rather unexpected result. However, it should not come as a complete surprise, as based on the structure of modern human DNA sequence variation, a contribution of more archaic humans to the modern human gene pool has previously been suggested (Plagnol and Wall 2006; Wall et al. 2009). Solely based on modern human DNA, the best estimates for genetic admixture from archaic hominids into the modern human gene pool were about 14% for Europeans and 1.5% for East Asians. Interestingly, these studies also find evidence for archaic admixture in Africa. Moreover, whereas the extent of admixture in East Asia roughly agrees with that suggested by the Neanderthal draft genome, for Europe the modern DNA data suggest an archaic contribution almost an order of magnitude larger than the Neanderthal genome draft does. Not surprisingly, the interpretation on the origin of these contributions is different between the studies. Wall et al., based on the modern DNA data, favor separate source populations in the different geographical regions with Neanderthals having contributed to the modern European gene pool and other archaic humans, like *H. erectus* or *H. floresiensis*, having contributed to the Asian gene pool. In contrast, Green et al. suggest a contribution of Neanderthals to both the European and Asian gene pool, because the signal of contribution is quite similar for modern humans from Asia and Europe. They suggest that gene flow took place

when humans and Neanderthals co-occurred in the Levant, during the colonization process of Eurasia by modern humans. In a recent comment, Hodgson et al. provide an alternative, but only slightly, different hypothesis. They argue that rather than during the colonization process of Eurasia, gene flow from Neanderthals into modern humans occurred earlier, about 100,000 yrs ago, and after this event the modern human range contracted back into Africa. Under this scenario, some traces of Neanderthal ancestry should also be detectable in Northern Africa. Given the rapid increase in the number of sequenced human genomes (Anonymous 2010), this hypothesis will soon become testable.

Thus, independent of the details of gene flow, there is increasing evidence that some gene flow took place between archaic and modern human populations. These results are neither compatible with a strict version of the recent African origin hypothesis that claims an exclusive recent African origin for modern humans without any contributions from archaic human populations, nor with the multiregional hypothesis that argues for close to equal contributions from archaic and modern humans to the current gene pool (see Stringer 2002 for a review of the various models). As it currently seems, the best model for modern human origins is replacement with limited gene flow, as previously suggested by some authors (Bräuer 1992). It should be kept in mind, though, that in absolute numbers, 2% contribution corresponds to as much as 120 million basepairs in each diploid Eurasian genome that would be of Neanderthal origin. However, there is a caveat to all these calculations that is noted in passing by both Green et al. and Wall et al. If substantial population substructure existed in the African gene pool, then no admixture from archaic human populations may be necessary at all to explain the data. Although additional data may allow us to distinguish between these alternative explanations, it is quite possible that we will never be 100% sure whether gene flow indeed took place between archaic and modern human populations.

Modern Human–Neanderthal Divergence. A second question that has major implications for our understanding of hominid evolution lies in the divergence time of Neanderthals and modern humans. Here one has to distinguish between genetic divergence and population divergence, with the former always, and sometimes substantially, predating the later. Since the publication of the first Neanderthal mtDNA sequences, researchers have tried to estimate the time when the two human forms separated. However, in contrast to the question of gene flow, the estimates for genetic divergence are remarkably consistent. They center around 600,000 to 660,000 yrs for mtDNA (e.g., Green et al. 2008; Endicott et al. 2010) and 825,000 yrs for nuclear sequences (Green et al. 2010). Using the nuclear data, Green et al. estimate the population divergence of modern humans and Neanderthals to 270,000 to 440,000 yrs BP. This timing is at odds with many interpretations of the fossil record, but for some reason Green et al. only mention this point very briefly and do not further discuss it. Interestingly, an analysis of the five complete mtDNA genomes from Green et al. (2008) and Briggs et al.

(2009) arrived at a quite similar population divergence, with the 95% confidence interval of the estimate being 315,000 to 538,000 yrs BP (Endicott et al. 2010). These dates are younger than most estimates of the divergence of modern humans and Neanderthals based on the fossil record, although a recent estimate using morphological characters arrived at a very similar age of 311,000 to 435,000 yrs ago (Weaver et al. 2008). These estimates have major implications for our understanding of hominid evolution.

Several models have been suggested to explain human evolution, mainly differing in the divergence time of modern humans and Neanderthals from a common ancestor (see Endicott et al. 2010 for a review of this topic). The genetic data now allow an independent estimation of the divergence time between these two hominids. A comparison of genetic divergence estimates and the various models on human evolution shows that several of these models propose divergence times much older than estimated by the DNA data. Thus, if the genetic estimates are correct, these models are relatively unlikely. Endicott et al. go so far as to suggest that only one model for human evolution, the mid-Middle Pleistocene model, which suggests a divergence of humans and Neanderthals from a common ancestor around 300,000 to 400,000 yrs, would fit the genetic data. Given the uncertainties of genetic dating, especially with regard to the calibration of the divergence which is done assuming a divergence time of humans and chimpanzees at 6 to 7 million years BP, this is probably quite a bold statement. However, the molecular dates do suggest that the interpretation of the human fossil record may require some major revisions. This is especially true for the interpretation of hominid fossils from Europe. Several fossils, such as the type specimen for *Homo heidelbergensis* or the hominids from Sima de los Huesos, which have been argued to fall on the evolutionary lineage of Neanderthals, actually pre-date the estimated divergence time for modern humans and Neanderthals. As discussed by Endicott et al. (2010), many of the dates estimated for hominid fossils are rather insecure. Therefore, it is also possible that the interpretation of these fossils is correct, and instead their assumed age is incorrect. Independently of whether the dates or the evolutionary assignment of these fossils is incorrect, some re-analyses of the hominid fossil record from both Europe and Africa is probably necessary. An excellent discussion of these issues has recently been published (Endicott et al. 2010).

The divergence estimates for humans and Neanderthals based on genetic data raise yet another issue, namely the question how many African–Eurasian dispersal events are required to explain the hominid fossil record. For this question, it is important to also take the recently published mtDNA sequence from Denisova into account, which diverges from human and Neanderthal mtDNA about 1 million years ago (Krause et al. 2010b). As pointed out above, there are various interpretations of the hominid fossil record, but discussing all of them would be beyond the scope of this paper. Rather, I will investigate how many dispersal events are required if mainly the genetic data available are taken

into account. The earliest dispersal of hominids out of Africa is indicated by the appearance of *Homo erectus* in Eurasia some 1.8 million years ago (Anton and Swisher 2004). Based on the archaeological context, Bar-Yosef and Belfer-Cohen (2001) proposed two more early dispersals from Africa, around 1.4 and 0.8 million years ago. Given the large confidence intervals of the genetic dating (780,000 to 1.3 million years), the unknown hominid from Denisova could be a descendant of either of these two waves. If the common ancestor of modern humans and Neanderthals lived in Africa, yet another dispersal event 300,000 to 400,000 yrs ago is required. Finally, modern humans emigrated from Africa some 50,000 to 60,000 yrs ago (Mcaulay et al. 2005; Fagundes et al. 2007), thus the total number of hominid dispersals from Africa would be at least five, and possibly more, which is clearly feasible. However, the genetic data can also be interpreted with as few as three African–Eurasian dispersals. In this scenario, after the initial dispersal by *Homo erectus*, no further early dispersals from Africa to Eurasia are required. Rather, as previously suggested (Bermúdez de Castro et al. 2008; Carbonell et al. 2008), the earliest European hominids from Sima de Elefante and Gran Dolina would have an Asian origin. Only two more African–Eurasian hominid dispersals would then be required, one from Europe to Africa some 300,000 to 400,000 yrs ago, resulting in the divergence of modern humans and Neanderthals and bringing the population ancestral to modern humans back to Africa and, finally, the dispersal of modern humans out of Africa. Although it should be pointed out that this scenario is quite speculative, it fits well with all genetic data, including the divergence date for the Denisova sample to modern humans and Neanderthals.

Taxonomic Implications. Similar to the issues discussed above, no consensus has so far been reached with regard to the taxonomic relationship of modern humans and Neanderthals. While some researchers argue that modern humans and Neanderthals represent distinct species (Harvati et al. 2004), others suggest that Neanderthals were rather a subspecies of our own species (Bräuer 2008). Distinguishing between specific, subspecific, or no taxonomic status at all is often already a controversial issue for living species, such as for example for African elephants (Roca et al. 2001; Debruyne 2005) or our closest relatives, the chimpanzees (Gagneux et al. 1999; Fischer et al. 2006). For extinct species, taxonomic status is even more difficult to decide, as one hallmark of species distinction, a lack of gene flow among them, can usually not be assessed from fossils. So given that the Neanderthal genome draft suggests that gene flow has taken place between modern humans and Neanderthals, does this show that they belong to the same species? Unfortunately, the answer to this question is no, as gene flow may well, and indeed regularly does, occur between what are by most scientists considered distinct species (Petit and Excoffier 2009). This is even true if they diverged several million years ago, such as fire-bellied toads (Szymura and Barton 1986). It should be noted that, given that humans and Neanderthals are large-bodied mammals with long generation times, the recent divergence time

between humans and Neanderthals argues against species-level distinction. However, in the end it remains a philosophical question whether the two human forms are assigned to the same or different species or subspecies, which is, moreover, largely irrelevant for understanding the process of human evolution.

Genes Selected on the Modern Human Lineage. Although the divergence among Neanderthals and modern humans has happened comparatively recently, 300,000 to 400,000 yrs is plenty of time for selection to act differently on the two lineages. Based on the fact that this divergence is recent enough that modern humans and Neanderthals often share derived single nucleotide polymorphisms (SNPs), Green et al. developed a test that allows screening for positive selection on the human lineage (for details of the method see Green et al. 2010). What is interesting about this method is that it is especially suited for screening for older selection events, thus many of the selective sweeps identified may have taken place relatively recently after the modern human–Neanderthal divergence. Using this test statistic, they identify 221 regions that were potentially under positive selection on the modern human lineage. The identification of such regions is not in itself surprising. Screens for recent local selection in modern humans regularly identify numerous genetic regions that show signs of positive selection (Lopez Herraez et al. 2009; Laland et al. 2010), although modern human populations separated a few tens of thousands of years at maximum. What is interesting, however, is the question which genetic regions were targets of selective sweeps on the human lineage. Potentially, these regions could be informative with regard to understanding in which ways modern humans and Neanderthals differed genetically and phenotypically.

Unfortunately, as is often the case in studies reporting whole genome screens, the results are quite vague. Of the twenty highest ranking regions, five contain no genes at all. Green et al. speculate that these "may thus contain structural or regulatory genomic features under positive selection during early human history." However, this is just rephrasing the fact that the cause of selection—if selection indeed acted on these regions—is unknown. The remaining fifteen regions contain between one and twelve genes. One region contains a gene potentially involved in type 2 diabetes, another one a gene that results, when affected by certain mutations, in skeletal defects. Finally, several regions contain genes potentially affecting cognitive traits. Although such speculations are no doubt interesting—and please human vanity that after all we may be more advanced in our cognitive abilities than Neanderthals were—there are several problems with these interpretations. First, most of the genes described have multiple functions, therefore the cause of selection could lie in any of these functions. For example, NRG3, highlighted by Green et al. as associated with schizophrenia, is also an important regulator of epidermal morphogenesis influencing epidermal and mammary gland development (Panchal et al. 2007) and involved in cancer development. These functions may be less glamorous than

an influence on cognitive traits but equally plausible as causes of selection. Second, as so far only a draft genome exists for Neanderthals, the test developed by Green et al. cannot be used for investigating which genomic regions were under selection on the Neanderthal lineage. Multiple high-quality Neanderthal genomes would be required to do so. It would no doubt be interesting to see whether, similar to the modern human lineage, many regions that are potentially involved in cognitive traits would show up as under positive selection on the Neanderthal lineage as well. And finally, it will be difficult to show the effect of any mutation or set of mutations specific to the human lineage. This is simply because of the fact that effects on cognitive abilities are hard to show in cell culture or the test tube, while the creation of transgenic humans carrying the Neanderthal version of a genetic region is unfeasible for both technical and especially ethical reasons.

Conclusions

The Neanderthal genome draft is not only a major technical accomplishment, it also raises a range of questions about human evolution. The genomic data provide the first convincing evidence that gene flow indeed took place between Neanderthals and modern humans, even though at a different time (earlier) and a different place (the Levant rather than Europe) than previously assumed. Molecular dating also shows that Neanderthals and modern humans diverged quite recently, a result that still needs to be fully reconciled with the fossil record. Finally, the elegant test for genomic regions under positive selection devised by Green et al. invites further speculations with regard to how Neanderthals may have differed from modern humans. What is required in the future are—perhaps not surprising—additional genomes. First from modern humans, especially from North Africa and Eurasia, to learn more about the timing and process of gene flow between humans and Neanderthals, and second, high-quality genomes from several Neanderthals. These later ones are important for at least two reasons. First, several high-quality Neanderthal genomes would allow testing for regions under positive selection on the Neanderthal lineage, which would be extremely interesting when compared to the regions under selection on the human lineage. And second, if the latest Neanderthal fossils are targeted (possibly in addition to some of the earliest anatomically modern humans from Europe), the question whether gene flow took place between these two human groups not only when they first met but also later on might eventually be settled. There can be little doubt that ancient human genomes have the potential to reveal many more interesting insights into human evolution.

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