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ARTICLE

Opsin gene repertoires in northern archaic hominids

John S. Taylor and Thomas E. Reimchen

Abstract: The Neanderthals' northern distribution, hunting techniques, and orbit breadths suggest that they were more active in dim light than modern humans. We surveyed visual opsin genes from four Neanderthals and two other archaic hominids to see if they provided additional support for this hypothesis. This analysis was motivated by the observation that alleles responsible for anomalous trichromacy in humans are more common in northern latitudes, by data suggesting that these variants might enhance vision in mesopic conditions, and by the observation that dim light active species often have fewer opsin genes than diurnal relatives. We also looked for evidence of convergent amino acid substitutions in Neanderthal opsins and orthologs from crepuscular or nocturnal species. The Altai Neanderthal, the Denisovan, and the Ust'-Ishim early modern human had opsin genes that encoded proteins identical to orthologs in the human reference genome. Opsins from the Vindija Cave Neanderthals (three females) had many nonsynonymous substitutions, including several predicted to influence colour vision (e.g., stop codons). However, the functional implications of these observations were difficult to assess, given that "control" loci, where no substitutions were expected, differed from humans to the same extent. This left unresolved the test for colour vision deficiencies in Vindija Cave Neanderthals.

Key words: Neanderthal, opsin, twilight, vision, genome.

Résumé : La distribution septentrionale des néandertaliens, leurs techniques de chasse et la largeur de leurs orbites suggèrent qu'ils étaient plus actifs en conditions de faible luminosité que les humains modernes. Les auteurs ont fait l'inventaire des gènes d'opsine chez quatre néandertaliens ainsi que deux autres hominidés anciens pour voir s'ils pouvaient apporter des évidences additionnelles en appui à cette hypothèse. Cette analyse était motivée par l'observation que les allèles responsables d'une trichromatie anormale chez les humains étaient plus répandus dans les régions septentrionales, par des données suggérant que ces variants pouvaient améliorer la vision crépusculaire et par l'observation que des espèces actives dans des conditions de faible luminosité possèdent souvent moins de gènes d'opsine que leurs parents diurnes. Les auteurs ont également cherché des évidences d'une convergence dans les substitutions d'acides aminés au sein des opsines des néandertaliens et de leurs orthologues chez des espèces crépusculaires ou nocturnes. Le néandertalien Altai, le dénisovien et l'homme d'Ust-Ishim (un des premiers hommes modernes) présentent des gènes d'opsines codant pour des protéines qui sont identiques à leurs homologues au sein du génome de référence humain. Les néandertaliens de la grotte de Vindija (trois femelles) présentaient plusieurs substitutions non-synonymes, incluant plusieurs qui sont prédites comme pouvant avoir un effet sur la vision des couleurs (p. ex. des codons stop). Cependant, les implications fonctionnelles de ces observations sont difficiles à mesurer, du fait que les locus de « contrôle », au sein desquels aucune substitution n'était attendue, se distinguent de ceux des humains d'une manière comparable. Ceci n'a donc pas permis de vérifier des déficiences quant à la vision des couleurs chez les néandertaliens de la grotte de Vindija. [Traduit par la Rédaction]

Mots-clés : néandertalien, opsine, mésopique, vision, génome.

Introduction

Humans are primarily daylight active and most have trichromatic colour vision (Neitz and Neitz 2011). The alleles in opsin genes that cause colour vision deficiencies (Jacobs 1981) increase in frequency northward from the equator (Birch 2012) and Reimchen (1987) proposed they might be beneficial in the low-light (mesopic) environments of high latitudes. This "twilight hypothesis" differed from the prevailing view that colour vision deficiencies (e.g., anomalous trichromacy) were maintained by mutation rate and relaxed selection in modern human environments (Post 1962). Since then, human color vision deficients have been shown to possess better contrast sensitivity at low light levels than "normal" trichro-

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mats (Verhulst and Maes 1998) while in marmosets (*Callithrix geoffroyi*), dichromats outperform trichromats when foraging at low light levels (Caine et al. 2010). Colour vision deficiency might also improve motion sensitivity in the dark (Srinivasan 1985). Simunovic et al. (2001) concluded that anomalous trichromats do not have superior night vision under scotopic conditions (e.g., moonless nights), but they recommended further study under mesopic conditions. Less controversial than the hypothesis that colour blindness is an advantage in the dark is the observation that some crepuscular and nocturnal species have reduced opsin gene repertoires compared to diurnal relatives (Bowmaker et al. 1994; Carvalho et al. 2006; Jackowska et al. 2007). Additionally, Paramei et al. (1998) found that there is a much smaller difference in wavelength discrimination performance between dichromats and trichromats during twilight. Thus, even if there are no benefits to mutations that cause colour vision deficiencies, their low cost might make them more common in dim light active species. We discuss opsin mutations in more detail below, but first we turn our attention to the Neanderthals.

Neanderthals appear to have been more active under dim light conditions than modern humans. Their range extended from central Europe into the Arctic Circle (Hublin and Roebroeks 2009; Slimak et al. 2011) where twilight is prevalent throughout the year and where winter day lengths are short. As well as co-existing with other top level predators (Anton et al. 2005), Neanderthals hunted medium- and large-sized ungulates, including woolly rhinoceros, reindeer, and mammoth and targeted adult-size classes (Richards et al. 2008; Bocherens 2009; Britton et al. 2011; Dusseldorp 2011; Niven et al. 2012). A common tactic of such top-level predators is to hunt at low light levels (Cooper 1990; Theuerkauf et al. 2003; Beauchamp 2007; Packer et al. 2011) because this allows increased proximity to prey and improved ambush (Reimchen 1998; Klinka and Reimchen 2009) and limits evasive options for the prey (Packer et al. 2011). Anatomical data from Neanderthals also suggest increased potential for low light activity. Relative to the size of the cranium, Neanderthals had larger orbits than early modern humans (EMH) (Pearce et al. 2013) and modifications to the inner ear indicative of increased sensitivity to both low and high frequencies relative to EMH (Kirk and Gosselin-Ildari 2009; Coleman and Colbert 2010). Similar modifications occur in a wide range of nocturnal mammals, including primates (Coleman and Boyer 2012). We hypothesized that data from opsins might provide further insight into Neanderthal life history.

We focused on "visual" opsins. In vertebrates there are five subfamilies; long wavelength sensitive or LWS opsins, medium wavelength sensitive opsins encoded by RH2 genes, short wavelength sensitive opsins belonging to either the SWS1 or SWS2 subfamilies, and RHO subfamily opsins expressed in rod cells. Gene duplication events within these subfamilies have generated some very large repertoires, especially in fish (Rennison et al. 2012). By contrast, the mammalian opsin repertoire has been reduced: Most mammals are considered to be dichromatic because they use RHO in dim (mesopic) light and SWS1 and LWS genes during the day. The "nocturnal bottleneck" hypothesis is the prevailing explanation for opsin loss in mammals (Walls 1942; Gerkema et al. 2013). Reversing this opsin-loss trend, trichromatic vision (i.e., daytime vision mediated by three distinct types of photoreceptors) evolved in the ancestor of great apes after a tandem gene duplication event generated a pair of opsins in the LWS subfamily. These paralogs, now called *OPN1LW* and *OPN1MW*, diverged at amino acid positions that influence wavelength sensitivity, the so-called five key sites (Yokoyama and Radlwimmer 2001). Mutations in *OPN1LW* or *OPN1MW* are the most common causes of human colour deficiencies: Null mutations in either gene lead to dichromatic vision, whereas anomalous trichromacy (e.g., protanomaly or deuteranomaly) occurs when gene conversion renders one more similar to the other at one or more key sites, thus reducing the difference between their peak wavelength sensitivities.

For reasons described above, we tested the hypothesis that deleterious mutations (e.g., premature stop codons or changes at key sites) in visual opsins were more common in Neanderthals than in humans, recognizing that we could expect to detect such an increase in the prevalence of colour vision deficiency causing mutations in the four female Neanderthals (eight X chromosomes) only if they occurred at a much higher frequency than 7%, which is the chromosome-level prevalence for colour vision deficiency in Caucasian humans. We also surveyed the Denisovan genome and the 45 000 year old Ust'-Ishim genome to more precisely map (on a phylogeny) any mutations we observed. This study represents a novel utilization of the archaic hominid genome sequences that to date have been used largely as outgroups to improve our understanding of human evolution.

Materials and methods

The Vindija Cave (Croatia) was the source of the first three Neanderthal genome sequences (Green et al. 2010). The DNA for these Neanderthals (all females) was obtained from bones estimated to be between 30 000 and 45 000 years old. The Altai Neanderthal genome (Prüfer et al. 2014) and the Denisovan genome (Reich et al. 2010) were obtained from DNA extracted from bones collected in the Denisova Cave in the Altai Mountains of southern Siberia. They were estimated to be between 30 000 and 50 000 years old. The bone that provided DNA for the 45 000 year old Ust'-Ishim man (the only male among these archaic genomes) was also collected in Siberia along the river Irtysh northwest of the Denisova Cave. See Fu et al. (2014, fig. 1) for a map showing Siberian sites.

The genome sequences studied here are comprised of reads (approximately 40 bp long) mapped by the original authors to the human reference genome (HG19). For the three Vindija Cave Neanderthals (Vi33.16, Vi33.25, and Vi33.26) and the Denisovan, read alignments on the UCSC Genome Browser were replicated in local multiple sequence alignments using BioEdit (Hall 1999). The original UCSC alignments were generated using ANFO, an application designed specifically to map short ancient DNA reads from Neanderthals to the human reference genome (https://bioinf.eva.mpg.de/anfo/). For the Vindija Cave Neanderthals, we removed nucleotide substitutions likely to have been caused by cytosine deamination (see Briggs et al. 2010). Specifically, we removed C→T substitutions if they occurred within five base pairs of the 5′ end of a read, and G→A substitutions within five base pairs of the 3′ end. This was prudent because the paucity of reads from these three samples meant that read-specific errors were not exposed by data from a large number of overlapping reads, which was the case for the Denisovan (and the Altai Neanderthal and Ust'-Ishim—see below). There was one exception to this substitution removal criterion: end-of-read substitutions that were also observed in the middle of an overlapping read were not considered to be errors. Next, we surveyed these local alignments for differences between the Vindija Cave Neanderthals, Denisovan, and human visual opsins that might influence dim-light sensitivity. We focused on premature stop codons and key site changes by translating the reads to identify nonsynonymous substitutions. We also compared these Neanderthal opsin sequences to orthologs from nocturnal species to see if there were any convergent amino acid substitutions, which would be candidate dim-light sensitivity adaptations.

For the Altai Neanderthal, read data from opsin-bearing chromosomes were obtained from the Max Planck Institute for Evolutionary Anthropology, Department of Evolutionary Genetics (http://cdna.eva.mpg.de/neandertal/altai/AltaiNeandertal/bam/). Focusing on one opsin-bearing chromosome at a time (i.e., chr. III, VII, and X), we used the SAMtools tview command (Li et al. 2009) and opsin gene exon coordinates (supplementary data, Table S1[1]) to survey exons for mutations. The Ust'-Ishim genome reads were not sorted by chromosome, so one file of mapped reads (i.e., Ust_Ishim.hg19_1000 g.all.bam) was obtained from the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/PRJEB6622) and surveyed using SAMtools tview. For these two samples the average read depth for all opsins (see Results) was high enough to allow us to identify errors and homozygous and heterozygous substitutions by eye in the output generated by SAMtools tview.

Gene copy number variation can be estimated from Next Generation Sequencing data. Alkan et al. (2009) used mrFAST to map short (36 bp) reads from three different humans to the reference genome and uncovered segmental duplications subsequently verified with qPCR. These analyses included the X-linked opsin gene array where read depth data exposed variation in *OPN1MW* gene copy number. In 2010, Green et al. (2010) demonstrated that such analyses could expose segmental duplications in low-coverage datasets, including a dataset assembled from the three Vindja Cave Neanderthals. The consensus genome they generated was shown to possess most of the segmental duplications characterized in humans, and in several cases copy number differed to a great extent between humans and Neanderthal. We used the SAMtools depth command to obtain per-position read depth values and calculated averages (and standard deviation) for each opsin from the Altai Neanderthal and the Ust'-Ishim by dividing the sum of position depth values by the number of bases covered. Note that for this calculation we included exon and intron positions. Read depth for the Denisovan and Vindija Cave Neanderthals are described qualitatively below.

Results

The data available for Vindja Cave and Altai Neanderthals differed to a large extent and for this reason we summarized these data and our efforts to evaluate reliability first (sections *i* and *ii*), and then report the results of our opsin gene analysis (sections *iii* and *iv*).

(*i*) Vindija Cave Neanderthal reads

The Vindija Cave Neanderthal opsin gene sequences were incomplete: The percentage of opsin gene positions characterized by reads for a given individual ranged from only 10.1% to 52.3% (Table 1; Data Files S1–S3[1]). Furthermore, the positions that were characterized were rarely covered by more than one read.

The Vindija Cave opsins differed from their human orthologs to a greater extent than the other samples. In addition, where Vindija Cave Neanderthal reads overlapped, they often differed from one another. Heterozygosity is one explanation for this last observation, but, for reasons discussed below, we attribute much of the variation between Vindija Cave Neanderthals and humans, and among and within Vindija Cave individuals, to sequencing errors.

To assess error prevalence in the Vindija Cave Neanderthal sequences, five control loci were surveyed. Control loci included *MECP2* and *RPA1* (both protein coding genes) and three ultra-conserved non-coding elements (UCEs), the *POLA1*-associated UCE, the UCE within *SFRS3*, and the *PBX3* intron UCE (Bejerano et al. 2004). Human

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2015-0164.

MECP2 and chimpanzee *Mecp2* genes encode identical amino acid sequences. Consequently, we expected the human and Neanderthal *MECP2* genes to be identical (or nearly identical). However, we found 17 *MECP2* substitutions, not including those attributed to cytosine deamination (see Materials and methods), among the three Vindija Cave Neanderthals (Table S2, Data File S4)[1]. This was 2.2% of the 782 bp considered. Two thirds (12/17) of these were nonsynonymous substitutions, a value expected if variation was random with respect to codon position, which would be the case for sequence errors but is unlikely to be the case for real substitutions in genes encoding such highly conserved proteins. Human and chimpanzee *RPA1* sequences (1851 nucleotides long) differed at 15 positions only, three of which were nonsynonymous changes, which is why it was also used as a control gene. *RPA1* genes from the three Vindija Cave individuals differed at 35 positions over a 1473 alignment (2.4% divergence) and a high proportion (21/35) were nonsynonymous substitutions (Table S2; Data File S5)[1]. In addition, reads from the three Vindija Cave Neanderthals covered 1845 bp (66.3%) of the 2782 bp of UCE sequence surveyed and differed at 38 positions (2.05%). By contrast, human and chimpanzee differed at 0.20% of the UCE positions aligned for these two species (Data Files S6–S8)[1].

In summary, the number of differences between Vindija Cave Neanderthal control genes and their human orthologs suggested approximately 2% of the variation observed in comparisons between human and the Vindija Cave Neanderthals was a consequence of imperfect DNA preservation or sequencing errors. This estimate did not include the end-of-read substitutions (which included 39 nonsynonymous changes) that were likely to have been caused by deamination (see Materials and methods).

(*ii*) Read data from the Altai Neanderthal, Ust'-Ishim, and Denisovan

For the Altai Neanderthal and Ust'-Ishim, all opsin exon (and intron) positions were characterized by overlapping reads. The average depths, which ranged from 19.5 to 57.8 reads per position, are reported with standard errors in Table 1. For the Denisovan, the percentage of positions characterized ranged from 89.4% to 100% (Table 1) and read depth was typically greater than five. Among these three genomes, only the Altai Neanderthal was surveyed at the control loci. The Altai Neanderthal *MECP2* gene was identical to the human ortholog (Table S2; Data File S4)[1], and the *RPA1* gene differed from human at one position; this individual was heterozygous at a third codon position (a synonymous change) that has been detected in other humans (Table S2; Data File S5)[1]. In addition, the Altai Neanderthal UCE sequences were identical to those from the human reference sequence (HG19) (Data Files S6–S8)[1].

(*iii*) Opsin gene sequences

Opsins from the Vindija Cave Neanderthals differed from their human orthologs at approximately 1% to 3% of the characterized positions (Table 1). This estimate did not include mutations thought to be caused by cysteine deamination, or two insertions excluded from the alignments because they inferred gaps in the human reference sequences that disrupted nucleotide to amino acid translations.

Substitutions were not distributed evenly among the opsin-aligned reads. Several were responsible for a disproportionate number of substitutions (e.g., reads M_SL-XAK_0004_FC30R25AAXX_6_94_170_387 and C_M_SOLEXA-GA04_JK_PE_SL19_repeat_3_59_419_517 together encoded 7 of the 15 vi33.16 *OPN1MW* nucleotide substitutions). Nonetheless, these multi-mutation reads had better MegaBlast scores when aligned to opsin genes than to any other region of the human genome, confirming that they had been correctly mapped on the UCSC genome browser. Additionally, some reads artificially enhanced sequence divergence at the amino acid level. *OPN1LW* and *OPN1MW*, as mentioned in the Introduction, are the products of a hominid-specific gene duplication event and are very similar to one another. As a result, some Neanderthal reads were as similar to *OPN1LW* as they were to *OPN1MW*. To avoid the artificial augmentation of human to Vindija Cave Neanderthal sequence divergence (i.e., at the amino acid level) we moved two reads: a 40 bp read from Vi33.16 mapped to human *OPN1MW* (C_M_SOLEXA-GA04_JK_PE_S49_repeat: 5:76:293:642) differed from *OPN1MW* and from *OPN1LW* at two nucleotide positions. We remapped it because these two differences inferred one amino acid change when mapped to *OPN1LW* and two changes when mapped to *OPN1MW*. The extra amino acid substitution when mapped to *OPN1MW* inferred an Ala285Thr substitution, which is predicted to influence the wavelength sensitivity. Another read, M_BIOLAB29_Run_ PE51_1:8:12:105:1186, which was from Vi33.25, differed from *OPN1MW* and *OPN1LW* at a single nucleotide position. It was aligned to *OPN1MW* on the Genome Browser where it also inferred an Ala285Thr substitution. However, when aligned to *OPN1LW* (and translated) it did not.

A total of 78 Vindija Cave Neanderthal opsin gene substitutions were observed (Table 1). However, only six were supported by more than one read. Two of these were within-individual substitutions. The first resulted in a V63I amino acid substitution in *RHO* from Vi33.16 (Data File S1)[1], and the second was a synonymous substitution in *OPN1SW* from Vi33.26 (Data File S2)[1]. Three substitutions supported by >1 read occurred among individuals. All of these were nonsynonymous substitutions that have been reported in humans (Data File S3)[1]. The sixth was the change at amino acid position 285 that was eliminated after read remapping (see above).

Table 1. Archaic hominid opsin gene sequence data.

	Neanderthals						
	Vindija Vi33.16	Vindija Vi33.25	Vindija Vi33.26	Vindija consensus	Altai	Denisovan	Ust'-Ishim
RHO							
Read count	18	9	11	38	849	84	612
Positions characterized	483	292	413	804	1047	1005	1047
Percent target characterized	46.1%	27.9%	39.4%	76.8%	100%	96.0%	100%
Average read depth (SD)					44.3 (10.9)		31.7 (13.6)
Positions w. syn. mut.	2	1	3	6	0	0	0
Positions w. nonsyn. mut.	3	1	4	8	0	0	0
Total	5	2	7	14	0	0	0
Identity	99.0%	99.3%	98.3%	98.3%	100%	100%	100%
OPN1SW							
Read count	18	18	16	52	887	77	785
Positions characterized	514	524	503	914	1047	983	1047
Percent target characterized	49.1%	50.1%	48.0%	87.3%	100%	94.0%	100%
Average read depth (SD)					49.2 (12.0)		36.6 (8.9)
Positions w. syn. mut.	1	3	2	6	0	1	0
Positions w. nonsyn. mut.	5	2	6	13	0	0	0
Total	6	5	8	19	0	0	0
Identity	98.8%	99.1%	98.4%	97.9%	100%	99.9%	100%
OPN1MW							
Read count	28	17	7	52	2391	204	825*
Positions characterized	573	287	111	740	1095	1095	1095
Percent target characterized	52.3%	26.2%	10.1%	67.6%	100%	100%	100%
Average read depth (SD)					57.8 (19.3)		19.86 (6.7)
Positions w. syn. mut.	2	1	2	5	0	0	0
Positions w. nonsyn. mut.	13	6	1	19	0	1	0
Total	15	7	3	24	0	1	0
Identity	97.4%	97.6%	97.3%	96.8%	100%	99.9%	100%
OPN1LW							
Read count	15	7	6	28	1010	80	376*
Positions characterized	410	208	267	632	1095	979	1095
Percent target characterized	37.4%	19.0%	24.4%	57.7%	100%	89.4%	100%
Average read depth (SD)					47.0 (8.0)		19.5 (7.1)
Positions w. syn. mut.	2	1	2	5	2	2	0
Positions w. nonsyn. mut.	7	3	5	13	2	2	0
Total	9	4	7	18	4	4	0
Identity	97.8%	98.1%	97.4%	97.2%	99.6%	99.6%	100%

Note: Read count, nucleotide positions characterized (total and percent of target gene), and substitutions (synonymous and nonsynonymous) reported for each Vindija Cave Neanderthal, for a Vindija Cave consensus genome (where reads from all three were evaluated as if from a single genome), and for the Altai Neanderthal, the Denisovan, and the Ust'-Ishim. Where the number of substitutions for the consensus genome is less than the sum of those for individuals (e.g., *OPN1MW*, positions with nonsynonymous substitutions), a given substitution occurred in two Vindija Cave Neanderthals. Average read depth and standard deviation calculated using samtools depth command (e.g., ./samtools depth –r X:153409758–153409869 ~/data/Neanderthal/chrX/AltaiNea.hg19_1000g.X.dq.bam | awk '{sum+=$3; sumsq+=$3*$3} END { print "Average = ",sum/NR; print "Stdev = ",sqrt(sumsq/NR – (sum/NR)**2)}'). The human reference genome (HG19) possessed two *OPN1MW* opsins that were almost identical. One began (the A in the ATG codon) at position 153448167 on the X chromosome and the other at position 153485285. Reads aligned to both loci were used in this study.

(*iv*) Opsin gene comparisons among archaic hominids and humans

Mutagenesis experiments have exposed eight amino acid positions that influence the RHO spectral sensitivity (Yokoyama et al. 2008). Data from Vindija Cave Neanderthals were available for six of these sites and they possessed the same residues as human. For *OPN1SW* there are 14 spectral tuning sites (Hunt et al. 2009). At nine of these positions, including the two that have the greatest influence on spectral sensitivity, the Vindija Cave Neanderthals had the same residue as the human reference sequence. For four key sites, Vindija Cave Neanderthals had either no data or an incomplete codon. At the last *OPN1SW* key site (position 90 in the bovine reference sequence) there were two Vi33.16 reads, one encoded a proline (P), which is the amino acid found in all other primates in our survey, and the other a serine (S). We surveyed SWS1 genes at NCBI and determined that the pig (*Sus scrofa*) also has an S at position 90. However, no

data are available that allow us to infer the consequences, if any, of this key site change.

As mentioned above, a key site change was observed among the *OPN1MW* reads from two Vindija Cave Neanderthals; however, these read were re-mapped to *OPN1LW* (eliminating the substitution) without a reduction in the alignment score. Four stop codons were also observed, one in *OPN1SW*, two in *OPN1MW*, and one in *OPN1LW*.

Our survey included comparisons with nocturnal species. A *RHO* mutation (N331D) in Vi33.26 was also observed in wolf and the wrinkle-lipped free-tailed bat. However, this change (if valid) is unlikely to be an adaptation to dim light vision because other nocturnal species (other bats, the loris, rat, and cats) have an N in this position. Lastly, we looked for amino acid substitutions in Vindija Cave Neanderthal *RHO* genes that might influence dim-light sensitivity as a consequence of their impact on all-*trans*-retinal release rates (Piechnick et al. 2012). None were observed.

The Altai Neanderthal, the Ust'-Ishim, and the Denisovan had opsin gene sequences that encoded proteins identical to those found in modern humans and the synonymous substitutions shown in Table 1 were common among the human opsin sequences available in the 1000 Genomes db (http://browser.1000genomes.org/index.html).

Copy number variation was anticipated as the number of opsins in the human X-linked *OPN1LW/OPN1MW* gene array varies to a large extent (Walls 1942; Terao et al. 2005; Verrelli et al. 2008). Altai Neanderthal appears to have had the same array as the human reference genome: one *OPN1LW* and two *OPN1MW* opsin genes (Table 1). There were fewer reads aligned to the X-linked opsins than from the two autosomal opsins in Ust'-Ishim (Table 1). This observation, approximately half as many reads aligned to *OPN1LW*, *OPN1MW1*, and *OPN1MW2* than to the autosomal opsins, is what would be expected in males, which have only one X chromosome. Read count data from the Denisovan (more than twice as many *OPN1MW* reads as *RHO*, *OPN1SW*, or *OPN1LW*) suggested that this genome included one *RHO*, *OPN1SW*, and *OPN1LW* locus and two *OPN1MW* genes (Table 1). Despite the success of the Neanderthal segmental duplication survey discussed above (Green et al. 2010), small differences in copy number (e.g., between any single Vindja Cave Neanderthal and the human reference genome) for a small region of the genome cannot be reliably estimated when the average read depth for that region is much less than 1.0.

Discussion

For extinct species, DNA sequences have the potential to enhance the perspective obtained from fossils alone. If we can identify their closest living relatives (e.g., by including ancient DNA in phylogenetic analyses) inferences can be made about morphology, life history, and behavior by assuming that extinct species looked and behaved like members of their extant sister group. In addition to including them in phylogenetic trees, occasionally specific genes are targeted that allow the identification of unique traits in extinct taxa. For example, the woolly mammoth hemoglobin gene has three derived amino acid substitutions that improve function at low temperatures and are thought to have been adaptations to range expansion from tropical climates (Campbell et al. 2010). Also, an allele at the *TRPM1* locus that causes spotting in extant horses has been detected in samples from the Pleistocene, suggesting that cave paintings depicting spotted horses are accurate reflections of the artists observations. Intriguingly, the discovery of this allele also suggests that "night blind" horses were fairly common at the time, heterozygotes for the *TRPM1* allele are spotted, homozygotes have congenital stationary night blindness (Pruvost et al. 2011). Similar work has been done on Neanderthals: PCR amplification and sequencing of *MC1R* from El Sidron Neanderthal DNA revealed alleles that influence pigmentation and led to the proposition that some were red heads (Lalueza-Fox et al. 2007) and the discovery that individual # SD 1253 (El Sidron) was heterozygous at *TAS2R38* (Lalueza-Fox et al. 2009) suggested that phenylthiocarbamide "non-tasters" were prevalent, at least in this population.

Neanderthal and Denisovan genomes were first published in 2010 (Green et al. 2010; Reich et al. 2010). A new Denisovan genome and a fourth Neanderthal genome became available in 2012 and 2013, respectively (Meyer et al. 2012; Prüfer et al. 2014). Although used primarily to identify human-specific alleles and, thereby, improve our understanding of human evolution, these resources also provide the opportunity to investigate ancient hominid morphology, behavior, and life history using a genetic approach. By reassembling read alignments for the three Vindija Cave Neanderthals and the Denisovan, and utilizing alignments provided in the .bam files for the Altai Neanderthal and the Ust'-Ishim, we compared their opsin sequences to those from modern human. We also compared them to other vertebrates that differ in the extent of diurnal and nocturnal activity. Our goal was to determine whether or not Neanderthals differed from EMH in a manner consistent with the hypothesis that they utilized a different photic environment. This search for unique features of Neanderthal genomes was a novel utilization of the data and it presented novel problems: Where Neanderthal data are used to further characterize differences between human and chimpanzee (e.g., to date human-specific alleles more precisely), sequence errors tend to be less of a problem because only positions where Neanderthal are the same as chimpanzee or the same as human and different from chimpanzee are considered. But, our interest in Neanderthal vision has led to the first study utilizing the publically available genome sequence data where Neanderthal-specific substitutions

were the target. We determined that the Altai Neanderthal, the Denisovan, and the 45 000 year old Ust'-Ishim had no unique nucleotide substitutions among their visual opsin genes and encoded opsin proteins identical to those in the human reference genome (HG19).

Although the number of substitutions we observed among the Vindija Cave Neanderthal opsins was only marginally greater than that observed among the control genes, there was an especially high proportion of nonsynonymous substitutions (including premature stop codons) among reads from Neanderthal Vi33.16 aligned to *OPN1MW* and a paucity of reads aligned to the *OPN1LW/OPN1MW* gene pair in all three individuals. It is not possible to determine how many copies of each opsin gene these Neanderthals had, but they did not appear to have possessed the large *OPN1LW/OPN1MW* gene arrays often found among humans. The possibility that Vindija Cave Neanderthals had null *OPN1MW* alleles is intriguing given that during twilight the visual light spectrum is bimodal, with the least irradiance in the middle region of the visible light spectrum (McFarland and Munz 1975; Johnsen et al. 2006; Veilleux and Cummings 2012). The admittedly limited evidence for *OPN1MW* gene degeneration is also interesting because, if valid, it infers a route to colour vision deficiency (and possibly enhanced dim light/long wavelength vision) that differs from humans. In humans, dichromacy and anomalous trichromacy, both of which are more common in northern latitudes, are usually a consequence of gene conversion events (i.e., mutations that make the X-linked opsins more similar to one another), not gene loss.

In summary, our analyses of the opsin sequence data in early hominids do not yet allow us to resolve the twilight hypothesis. While we found suggestive evidence for opsin gene mutations in the Vindija Cave Neanderthals, the high quality sequences of the Altai Neanderthal, the Denisovan, and the Ust'-Ishim are effectively identical to a modern human trichromat. When high quality genome sequence data are available for a larger sample of archaic hominids, it will become possible to better assess the frequencies of colour deficiencies.

Author contributions

T.E.R. conceived this project, and J.S.T. and T.E.R. analyzed and interpreted the read data.

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