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SPY CAVE

125 years of multidisciplinary research
at the Betche aux Rotches
(Jemeppe-sur-Sambre, Province of Namur, Belgium)

Edited by Hélène ROUGIER & Patrick SEMAL

Volume 1

2013

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CHAPTER XVIII

MOLECULAR TAPHONOMY OF SPY: DNA PRESERVATION IN BONE REMAINS

Eva-Maria GEIGL, Sophie CHAMPLOT, Silvia DE LIMA GUIMARAES, E. Andrew BENNETT & Thierry GRANGE

Abstract

Palaeogenetic studies of faunal remains from archaeological sites can provide invaluable information if DNA is preserved. The quantity and quality of the genetic information that can be retrieved critically depends on the biomolecular preservation in the fossils. Here we report the assessment of the preservation state of DNA in faunal and human remains of the cave of Spy. Using a quantitative PCR approach we found that DNA fragments up to 150 bp were preserved at low quantities in half of the analysed bone samples belonging to four different taxa. The prevalence of deaminated cytosines was found to be low. We discuss the results in light of common knowledge of in vivo DNA degradation and preservation.

INTRODUCTION

Genetic analyses of ancient fossil material can provide invaluable information on the evolution of species, including that of humans, on the interpretation of archaeological sites and on palaeoenvironments. The success of such an approach, however, critically depends on the chemical preservation of DNA. A better identification of the conditions favourable for DNA preservation would allow proper assessment of the chances of success of a palaeogenetic approach and would enlighten the decision of subjecting precious fossil material to a destructive analysis. Human and animal remains in the cave of Spy are morphologically well preserved. In order to maximise the information that can be retrieved from this archaeological site and thus increase the accuracy of its environmental and biological interpretation, it is highly desirable to also perform genetic analyses of these remains. Although morphological and DNA preservation are not directly linked (unpublished observations of the authors), a well-preserved histology of fossilising bones can be a positive indicator for DNA preservation (Colson *et al*., 1997). To reliably assess the potential of these bones for palaeogenetic analyses, we performed a study of the preservation state of DNA in faunal remains from Spy on the way of a potential palaeogenomic analysis of certain precious remains. Here, we will discuss the mechanisms of DNA degradation and the challenges faced by palaeogenetic studies and we will present our preliminary results on DNA preservation in several of the fossils of the cave of Spy.

DNA – the molecule of life

Deoxyribonucleic acid, DNA, is the molecule of life since it contains the information for the morphological and functional features of an organism and for its development. This information is divided into two main parts, one coding for the structural information that translates into the synthesis of structural and functional proteins, one coding for the regulatory information controlling the timing, location and levels of protein synthesis throughout the development and in the adult.

DNA consists of long strands of sugar molecules called deoxyribose that are linked together by phosphate groups. Each sugar molecule carries one of the four natural DNA bases, i.e. adenine, guanine, cytosine, or thymine (A, G, C, or T). These DNA bases constitute the genetic code comparable to the letters in an alphabet. The combination of three of these DNA bases codes for an amino acid, the monomeric units which make up proteins.

Ancient DNA analysis

In the 1980s it was discovered that low quantities of short DNA molecules can be preserved in calcified tissue in specific conditions that are still not very well understood. The newly developed method of PCR (polymerase chain reaction) allowed the multiplication and analysis of a few preserved molecules (see below). This methodological development not only revolutionised molecular biology but led also to the birth of the field of palaeogenetics. Over the years, studies of extinct organisms increased exponentially (for a review, see Pääbo *et al.*, 2004). They all were based on the analysis of a short mitochondrial DNA sequence that mutates very quickly, the hypervariable region or D-loop. Since each cell contains up to 8,000 copies of the mitochondrial genome, the likelihood to find preserved molecules is higher than for the two nuclear copies of a given sequence in a diploid nuclear genome. For almost twenty years, these features made the mitochondrial D-loop the sequence of choice for palaeogenetic analyses. They contributed to the phylogenetic and phylogeographic analysis of extinct species, to the elucidation of the domestication processes of various animals and plants, and to past human and animal migrations (for a review, see Pääbo *et al.*, 2004).

Since then, the newly born field of palaeogenetics made enormous methodological progress benefiting from the advances of molecular biology that experienced a development at a breathtaking pace. This can be seen at best through the fact that already the entire genome of two single individuals, each composed of 3 billion base pairs, can be sequenced in only eight days. Soon we will know the sequence of 1,000 human genomes (http://www.1000genomes.org), the study and comparison of which will allow a deep insight into the biological evolution of our species. These advances made it possible to also analyse genomes from extinct species characterised by an exceptional preservation. In particular, the sequence of the entire genome of a mammoth preserved in the Siberian permafrost was published in 2006 (Poinar *et al.*, 2006). More recently, the genome of an ancient Eskimo (Saqqaq) was elucidated using a hair sample (Rasmussen *et al.*, 2010). Most importantly, the genomic sequence preserved in bones from a cave in Croatia (Vindija) and belonging to *H. neanderthalensis* was published (Green *et al.*, 2010). Finally, the genome of an archaic human population, formerly unknown to palaeoanthropologists, that inhabited Southeast Asia around 40,000 years ago, the so-called Denisovans, was elucidated (Krause *et al.*, 2010; Reich *et al.*, 2010). The analyses of both of these genomes have shown the occurrence in the past of admixture between Neandertal, modern and Denisova humans (Green *et al.*, 2010; Reich *et al.*, 2010).

Despite these enormous successes, a number of problems remain that are intrinsic to the studied material, ancient DNA, the first one being contamination with modern DNA molecules (e.g. Champlot *et al.*, 2010), the second being nucleotide base damage (Briggs *et al.*, 2007; Green *et al.*, 2009).

ANALYSIS OF THE DNA IN REMAINS FROM SPY

Analysis of the preservation state of DNA

We were interested in characterising the chemical preservation state of DNA in the abundant and morphologically well-preserved faunal remains from Spy. This study was performed on animal remains instead of human remains since the latter ones are the most susceptible to contamination with modern human DNA. Modern human DNA is also the most abundant type of contamination during excavation, exhumation of the bones and teeth, as well as during palaeontological and palaeogenetic analysis.

Using approaches of molecular biology and in particular quantitative real-time PCR (Pruvost & Geigl, 2004), we analysed the quantity of mitochondrial DNA preserved in faunal remains from various species. Moreover, we analysed the fragment length of the preserved DNA molecules by targeting DNA fragments of various lengths. Finally, we analysed the quantity of deaminated cytosines contained in the ancient DNA templates.

Through the use of DNA-binding fluorescing dyes during the PCR it is possible to follow in real-time the synthesis of new DNA molecules

and to quantify the number of initial target molecules (for a review, see Lutfalla & Gilles, 2006). At the same time this allows the identification of PCR parasite products such as primer-dimers, the formation of which exhausts the pool of reagents and interferes with the amplification and detection of small numbers of initial molecules (Chou *et al.*, 1992). We optimised this procedure, called qPCR, for the analysis of low copy number DNA using buffer conditions in which the Taq polymerases have the highest fidelity (Pruvost *et al.*, 2008). Furthermore, we use dUTP during PCR and uracil-N-glycosylase (UNG) to degrade potential traces of carry-over contaminating molecules from previous reactions that would have escaped the physical containment procedures (Pruvost *et al.*, 2005). Finally, we measure the inhibition potential of each fossil extract (Pruvost & Geigl, 2004), and identify PCR conditions that minimise interference between the fossil extract and the PCR reagents in order to achieve optimal PCR efficiency and fidelity for every extract analysed (Pruvost *et al.*, 2005, 2008).

Using this low mutagenic, highly sensitive and contamination-minimising UQPCR procedure, we amplified different parts of the hypervariable region of the mitochondrial DNA from the extracts of 14 bone remains from Spy. In particular, we analysed 11 equid bones/teeth, one reindeer bone, one mammoth tooth and one human bone (see Table 1). We obtained DNA sequence information from five out of 11 equid bone/tooth extracts, as well as from the reindeer bone and the mammoth tooth extract.

The DNA from five of the 11 analysed equid remains could be amplified. These were a metacarpal, excavated in 1885/6 by Lohest & De Puydt and dated to more than 36,000 and less than 50,000 years, and four teeth, excavated in 1952/3 (see Table 1). The amplified fragments were 83, 89, 105, 110, 145 and 154 nucleotides long and covered the region 15,724 to 15,834 of the hypervariable mitochondrial DNA. They all were caballine sequences that cluster with modern *E. caballus* sequences. Thus, they fall into the genetic diversity of modern horses.

The metatarsal of a reindeer (*Rangifer tarandus*), excavated in 1952 and dated to 34,410 +230/-210 BP (see Semal *et al.*, this volume: chapter XVI), was preserved with ochre. We could amplify a 98 and a 127 nucleotide-long fragment of the mitochondrial cytochrome oxidase gene in regions 7,001 to 7,098 and 8,541 to 8,667. Each fragment differs by one substitution from the sequence's closest living relative in the sequence database, a reindeer from Japan (GenBank accession number AB245426, see Table 2;

Species	*Code*	*ID*	*Skeletal part*	*Stratigraphy*	*Excavation*	*aDNA results*
R. tarandus	SPY-D4	Spy D4 19B 121 1480 MT 40126	Metatarsal frag.	*Déblais sup.* / 14 A-E	1952	yes
R. tarandus	SRa3	Spy 19B 122 1489	Astragal	*Déblais inf.* / 17 A-E	1952-1954	no
Equus	ULg 10	Spy 10091 ULg	Metacarpal		1885-1886	yes
Equus	SEq1	Spy 19B 121 1472 (échantillon 1)	M3	*T.H.* / 27 D-E / 0.5-0.8	1953	no
Equus	SEq2	Spy 19B 121 1472 (échantillon 3)	M3	*T.H.* / 27 D-E / 0.5-0.8	1953	no
Equus	SEq3	Spy 19B 122 1498 IG 18921 (échantillon 4)	M3	*Tr. C* / 26 C / 0.5-0.7	1953	no
E. hydruntinus	SHyd1	Spy 19B 122	Upper P3/M2	*Déblais inf.* / 18 A-E	1953	no
E. hydruntinus	SHyd2	Spy 19B 122	Tooth	*Tr. C* / 27 C / 0-0.8	1953	yes
E. hydruntinus	SHyd3	Spy Liège 2B 14038	P2	*Niveau inférieur/rouge*	1885-1886	no
E. hydruntinus	SHyd4	Spy 19B 121 1470	Lower P3/M2	*Déblais inf.* / 13 A-E	1952-1954	yes
E. hydruntinus	SHyd5	Spy 19B 121 1478 IG 17393	Upper I3	*Déblais surf. anc. sol* / 1-14 D/E	1952-1954	yes
E. hydruntinus	SHyd6	Spy 19B 121 1474	Upper left P2	*Déblais sup.* / 23 A-E	1952	no
E. hydruntinus	SHyd7	Spy 19B 121 1480	Lower M3	*Déblais sup.* / 14 A-E	1953	yes
M. primigenius	SPY-D3	Spy D3 19B 121 1474 40129	Lower m2	*Déblais sup.* / 23 A-E	1952	yes
H. s. sapiens	SPY-HS	Spy 425k	Right fibula frag.	*T.H., surface anc. sol* / 1-14 D/E	1952	no

Table 1. Bone samples analysed in the present study using a quantitative palaeogenetic approach.

		Reindeer		Mammoth	
Upper Primer	Name	R1	R9	M15	M17
	Sequence	ACCCGTACAC CTCATATGGCT	TTCGCAGTAGC TATGATTCAGG	GCTCTTGATCGT ACATAGCACATT	CTTAACTACCTA CCTCCGAGAAAC
Lower Primer	Name	R2	R10	M16	M18
	Sequence	AGGGTGTGATC ATGAAAATGTAA	GTTAGGGGTCAGGGGCTT	GATGGGCGGGTTGATGG	ATCCAGTATAG AAACCCCCACA
PCR product	Position, including primers	7,001-7,098 (AB245426)	8,541-8,667 (AB245426)	15,679-15,789 (EU153449)	15,750-15,845 (EU153449)
	Sequence, excluding primers	TATCCAATACAACTAGGC TTCCAAGATGCAACATCA CCTATTATAGAAGAACTA	CATATGTATTTACCCTTC TAGTTAGCCTCTACCTG CATGACAACACATAATG ACACACCAAACTCATG CCTACCATATAGTAAATCC	ACTGAGAAATCTCTAGTC ACCATGCATATCACCTCC AACGGTTGTACCTTAACT ACCTACCTCCGAGAAA	CATCAACCCGCCCATCT TCGTGTCCCTCTTCTCG CTCCGGGCCCATCAAT
With UNG	Number bases sequenced without primer	325		358	
	Number GC sequenced	116		196	
	Number transition C>T, G>A	0		0	
	%GC mutated	**0**		**0**	
Without UNG	Number bases sequenced without primer	456		357	
	Number GC sequenced	177		183	
	Number transition C>T, G>A	1		3	
	%GC mutated	**0.6**		**1.6**	

Table 2. Nucleotide sequences of the PCR primers and PCR products of the reindeer and mammoth analysed and number of deaminated cytosines detected. Deaminated cytosines show up in PCR products as guanine to adenine (G>A) and cytosine to thymine (C>T) substitutions.

Benson *et al.*, 2008). An identical sequence was obtained from reindeer remains of the Mousterian site of Les Pradelles, Poitou-Charente, France, contemporaneous to those of the site of Spy (Maureille *et al.*, 2007).

We also analysed the tooth of a juvenile mammoth (*Mammuthus primigenius*) dated to 42,330 +550/-450 BP and excavated in 1952. It contained amplifiable DNA fragments of 95, 96, and 111 nucleotides. The mammoth sequences stretch from positions 9,111 to 9,205 of the cytochrome C oxidase subunit III gene, and from positions 15,679 to 15,789 and 15,750 to 15,845 of the hypervariable region of the mitochondrial D-loop sequence of a Siberian woolly mammoth (EU153449, see Table 2). While all three of these sequences match 100 % *M. primigenius* sequences in Genbank (in 2013), the latter two sequences also match 100 % existing elephant species and are therefore not specific enough for species identification. However, the 95 nucleotide cytochrome C oxidase sequence was found to be specific to woolly mammoth to the exclusion of other elephant and mammoth species. Thus, we confirm genetically that this tooth indeed belongs to *M. primigenius*, which is compatible with its existing range at the time.

As a general tendency, for those bone extracts that yielded amplification products, longer amplicons (roughly more than 100 bp) were obtained less frequently than shorter ones. A strong quantitative correlation, however, could not be established, which might be a consequence of the fact that the number of molecules analysed was generally below five, and thus too small to permit robust quantification of molecules because of statistical fluctuations.

No species-specific DNA preservation could be established, which might be simply due to insufficient sample size, since we analysed only one sample from *M. primigenius* and one from *R. tarandus*, and they both yielded amplification products, whereas eleven equid samples were analysed from which only five could be amplified. This result lacks statistical support.

We also analysed alterations of nucleotide bases occurring during the course of diagenesis, in particular deaminated cytosines, the major lesion type observed in ancient DNA (Hofreiter *et al.*, 2001; Briggs *et al.*, 2007). Deaminated cytosines can be recognised and removed using UNG, as mentioned above (Lindahl, 1979). The removal of a deaminated cytosine by the UNG leads to a strand break, which, if occurring within the sequence bracketed by the two primers used, should remove the corresponding DNA molecules from the pool of the PCR-amplifiable templates. Thus, the UNG-dependent loss of amplifiable molecules should be observable if the frequency of deaminated cytosines found in the targeted region is sufficient.

We took advantage of these two features to determine the frequency of the occurrence of deaminated cytosines. On one hand, we analysed the quantity of amplicons produced in the presence and in the absence of UNG, and on the other hand we analysed the occurrence of base transitions in the amplicons. In the case of the mammoth and the reindeer bone analysed for this feature in the present study, the amplifications in the presence of UNG yielded in general more amplification products than in the absence of UNG. We, as well as others (Orlando *et al.*, 2006), have observed a similar phenomenon using other bones. This suggests that, not only the frequency of deaminated cytosines in central regions of DNA molecules is not high enough to remove a detectable amount of template, but also that some additional template molecules could be rendered available for PCR amplification following UNG cleavage. One possibility is that UNG could release cross-linked molecules that otherwise could not participate in the PCR. It is, in particular, possible that the release of DNA molecules shorter than the full length template could produce amplifiable molecules following several PCR cycles in a process called “jumping PCR”, involving the use of partially extended primers as new primers in subsequent PCR cycles (Pääbo, 1989).

When the amplification products of the same bone/tooth extracts, i.e. from the mammoth tooth and the reindeer bone, were sequenced, C/T transitions, the results of the deamination of cytosines followed by amplification with Taq DNA polymerase, were observed in the amplicons obtained without UNG digestion prior to PCR. In contrast, after UNG treatment, no C/T

transitions were observed (see Table 2). Cloning was not necessary to detect mutations due to deaminated cytosines because most PCR products were obtained from either a single or at most two starting molecules, as judged from qPCR quantifications, and base substitutions can be detected when the ratio of parental to substituted base is 1:1 (Pruvost *et al.*, 2008).

Independent PCR amplifications of DNA fragments between 96 and 127 base pairs were performed from two different reindeer and mammoth extracts, respectively. Each extract was either untreated or treated with UNG prior to amplification and a number of independent PCR products were sequenced in each case. The PCR products obtained in the absence of UNG treatment using the reindeer and the mammoth extracts contained mutations involving C>T or G>A transitions affecting only 0.6 and 1.6 % of Gs and Cs, respectively (Table 2). In contrast, no such mutations were detected following UNG treatment of the extracts prior to the PCR. This is consistent with the exceptionally low rate of such substitution that we encounter routinely using UNG treatment (Pruvost *et al.*, 2008).

To conclude, the frequency of the occurrence of diagenetic lesions, i.e. deaminated cytosines, in the DNA preserved in a mammoth tooth and a reindeer bone from Spy is relatively low when analysing their DNA using PCR. The actual frequency, however, might be higher. In fact, using next generation sequencing technology it has been observed that the highest incidence of this type of lesion occurs at the ends of DNA fragments (Briggs *et al.*, 2007; Green *et al.*, 2009). Standard PCR only amplifies the continuous DNA sequence between where the two primers hybridise, thus the ends of DNA fragments are not amplified. Since the ends of PCR products are derived from the primer molecules and not the target molecule, instances where a primer hybridises to the very end of a fragment will also mask any lesions occurring there. Thus, most deaminated cytosines would not be detected using standard PCR.

We therefore compared the results of our PCR experiments described above with those obtained from next generation sequencing (Illumina) of mitochondrial DNA in a reindeer extract from the French Mousterian site of Les Pradelles in the absence of prior UNG treatment of the extract. Here we found 50 C>T and G>A transitions in the 890 nucleotides sequenced that were located at a distance of 5 nucleotides from the ends, corresponding to 320 Cs and Gs (data not shown). Thus, 15.6 % of the Cs and Gs were mutated. In contrast, the central parts of the corresponding fragments, corresponding to 2225 nucleotides analysed, including 801 Gs and Cs, contained only 4 transitions, i.e. a value of 0.5 % that is very similar to that detected using standard PCR. Thus, the rate of cytosine deamination appears to be 30 times higher at the ends of the ancient DNA molecules than in the centre. This is presumably due to the single-stranded nature of the ends because cytosine deamination rate is much higher in single-strand DNA in agreement with previous interpretations (Briggs *et al.*, 2010).

Finally, DNA is rather well preserved in the animal remains from Spy, which were discovered in a layer of hematite, a black to brick-red mineral, essentially Fe_2O_3, the chief ore of iron (see Goemaere *et al.*, this volume: chapter VIII). It would be interesting to analyse whether the hematite layer that preserved the animal remains and that presumably was used by the Aurignacians to protect the animal carcasses from scavengers had an effect on DNA preservation. This could be done either by analysing *in vitro* the effects of Fe_2O_3 on DNA stability or by comparing DNA preservation in bone remains from the site that did not experience this treatment. Indeed, there could be a dual effect of iron because enhanced production of hydroxyl radicals due to the iron catalysed Fenton's reaction would promote the inhibition of bacterial growth on one hand and DNA degradation on the other hand.

Analysis of the post-excavation treatment

The human remains from the Lohest collection (1886) conserved in the Royal Belgian Institute of Natural Sciences (RBINS) are known to have been varnished, probably when they arrived at the RBINS in the '30s. For this reason they were not proposed for a palaeogenetic analysis. Instead, human bones that were conserved at the *Université de Liège* and thought not to have experienced this consolidation treatment were subjected to ancient DNA analysis. In

2000, at the Ludwig-Maximilians University (Munich, Germany), the amplification with mitochondrial D-loop primers of an extract from the right scapula Spy 572a (31,810 ± 250 BP, GrA-21546; Toussaint & Pirson, 2006), in the hands of Matthias Krings, yielded first a mitochondrial goat sequence, and after a modification of the extraction protocol also a mitochondrial human, possibly Neandertal, sequence (M. Krings & P. Semal, pers. com.). From the difference in amplification success it was concluded that the bone must have been contaminated with a relatively high quantity of goat DNA.

The origin of this contamination is unclear. It could come from (i) carry-over contamination in the laboratory, where in the first case modern goat was amplified just before the amplification of the Neandertal bone was carried out. Alternatively it could be the result (ii) of a contamination on the excavation site by modern goat, such as feces or urine, or (iii) of a particular post-excavation treatment, such as washing of several bones together, including goat bones. Alternatively, it could be due to a chemical agent with which the bones were treated and that had been prepared from animal tissue. Indeed, we think that the human bones from the *Université de Liège* may have been consolidated with glue. From historical sources it is known that glue could be made out of goat (see SI1). This could also explain the young radiocarbon date obtained on the Spy 572a scapula (Semal *et al.*, this volume: chapter XVI).

To retrace the contamination history of these bones (hypotheses i, ii or iii), we analysed several specimens identified in the faunal collection (not varnished or consolidated) with primers that are specific for goat mitochondrial DNA. They are a Neolithic human bone from Spy (Spy 425k, dated to 4,350 ± 35 BP; Semal *et al.*, this volume: chapter XVI) and some of the equid remains. Various extracts of the bone sample of a Neolithic human did not yield any amplification product, neither with the sheep nor with the goat-specific primers in a total of 32 PCRs. Finally, the same was true for three equid samples. Thus, we can conclude that neither of these bones coming from different "fauna-bearing levels" of the cave were contaminated in situ (in the site) with goat DNA.

At the same time, we analysed a piece of dry resin that, according to Marcel De Puydt's family, is supposed to have been used to prepare the glue (or varnish) with which the bones were treated. No amplification product was obtained in a total of 81 PCRs with the purified resin extract, neither using sheep-specific primers amplifying a 65 and a 125 bp long DNA fragment, nor goat-specific primers amplifying a 97 and a 112 bp-long DNA fragment. We cannot rule out, however, that the DNA trapped in the resin that was fabricated at the end of the 19th, beginning of the 20th century, is completely degraded by now.

Thus, the tested resin is probably not the source of the contamination but we cannot exclude that the human bones were treated with another product contaminated with goat DNA that was not available for testing.

CONCLUSION

In conclusion, the ancient DNA analysis via PCR of faunal remains from Spy showed that small quantities of authentic endogenous DNA molecules with a fragment length of 100 bp on average are preserved in most of the bone fragments and that the frequency of the occurrence of deaminated cytosines was not very high. Thus, preservation conditions for DNA in the bones preserved in the cave of Spy are relatively favourable.

PCR can detect single molecules for reasonable experimental costs and qPCR can allow estimation of the amount of molecules present. In contrast to NGS of the DNA in the fossil extract, it does not provide, however, information about the average size of the DNA fragment preserved, the extent of contamination with DNA from other sources (bacteria, fungi, other environmental DNA) and the damages at the ends of the fragments. Thus complete molecular characterisation of the diagenetic transformations of the DNA and of the correlation of DNA preservation and the taphonomy of the fossils requires additional studies. Because of the important costs that are still necessary to perform large-scale sequencing, it is presently reasonable to use these NGS approaches only to

analyse fossils for which it is most useful to obtain genomic information (i.e. Neandertal bones for the Spy fossils).

However, dramatic cost decreases in the future should render these analyses feasible on less precious material and allow a more thorough systematic characterisation of the diagenetic transformations of DNA and a better understanding of molecular bone taphonomy and the key parameters of long-term DNA preservation.

Finally, this series of experiments renders unlikely that the contamination on the excavation site by modern goat, such as feces or urine, is responsible for the goat DNA that was observed in the analysis of the Neandertal bone from Spy mentioned above.

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