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Aspartic Acid Based Aminostratigraphy of Spanish *Ursus deningeri* von Reich. and *Ursus spelaeus* Ros.-Hein. Localities

by

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Abstract

The results of amino acid racemization (aspartic acid) ratio analysis from the dentine collagen of cave bear species from Europe: *Ursus deningeri* von Reichenau and *Ursus spelaeus* Rosenmüller-Heinroth are presented. Using the extent of aspartic acid racemization it has been possible to formally establish for the Iberian Peninsula, an *Ursus deningeri* aminozone, whose ratios do not significantly overlap with *Ursus spelaeus*. It is evident that both species are not a case of polytypism but have a real chronostratigraphic significance. The short pawed cave bear, *Ursus spelaeus parvilatipedis*, represents a later subspecies, which has also chronostratigraphical value. Therefore two *Ursus spelaeus* subaminozones have been also defined.

Zusammenfassung

Es werden die Ergebnisse einer Studie über Aminosäuredatierungen von Aspartam aus dem Kollagen des Dentins von Vertretern der Höhlenbären-Gruppe vorgestellt. Anhand des Razemisationsgrades von Aspartam war es möglich, auf der Iberischen Halbinsel eine *Ursus deningeri*-Aminzone zu postulieren, deren Verhältniszahlen sich nicht signifikant mit denen von *Ursus spelaeus* überlappen. Es ist augenscheinlich, daß beide Arten nicht einen Fall von Polymorphismus darstellen, sondern einen tatsächlichen chronostratigraphischen Aussagewert haben. Der Kurztatzenbär, *Ursus spelaeus brevilatipedis*, ist eine später entstandene Unterart, die ebenfalls von chronostratigraphischem Wert ist. Daher wurden auch zwei *Ursus spelaeus*-Subaminozonen definiert.

1. Introduction

The dating of Quaternary fossil bear remains has been considered one of the fundamental objectives for palaeontogist but the lack of materials suitable for radiometric dating constituted a very important constraint making very difficult to ascertain the stratigraphical value of the aforementioned species, some subspecies too, sometimes interpreted as a polytypism-linked phenomena.

Trying to solve it since 1994 the Laboratory of Biomolecular Stratigraphy of the Madrid School of Mines has been working to develop a dating method based on amino acid racemization in bear teeth dentine. We present here the first obtained results which demonstrate that amino acid racemization dating from cave bear dentine collagen is a proxy method for dating. The method basis lies on the fact that in living beings, with only some exceptions, there are only L-amino acids; after the organism's death the racemization process begins. Racemization is a first order chemical reaction, which transforms L- amino acids into D-amino acids until the D/L ratio equals 1 meaning that the final racemic stage is reached. In amino acids with asymmetrical binds (such as isoleucine) the transformation of the L-enantiomer into de D-enantiomer is called epimerization, since these D an L compounds are not mirror images and, in this case, the D/L ratios can reach values greater than 1 (1,4 for D-*Allo*/L-

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isoleucine). In long life living beings, as man, amino acids begin to racemize throughout the life of the organism (JOHNSON & MILLER, 1997) and have been employed in forensic determinations (OHTANI et al., 1988)

The kinetics equation is:

$$\ln\left(\frac{1+D/L}{1-D/L}\right) - C = (1+K')\,K_L\,t$$

t: time

$K' = K_D / K_L$

D/L ratio was obtained from GC or HPLC analyses.

C is the sample preparation method induced racemization.,

K_L and K_D have been found through laboratory thermal induced racemization, see WEHMILLER & BELKNAP (1982); RUTTER & VLAHOS (1988) and GOODFRIEND & MEYER (1991).

Because of their abundance we have chosen to work with aspartic acid racemization ratios.

Racemization ratios values can be employed not only for aminostratigraphical purposes, as in this work, but also for aminochronological purposes.

Amino stratigraphy consists on establishing a stratigraphical order of a number of paleontological localities with the same thermal history, as in most of the Iberian localities happens. Since the amino acid racemization method is not an absolute dating method, it is necessary to work with previously calibrated (throughout radioactive dating methods as U-series, ESR or ^{14}C) samples to obtain age calculation algorithms of local (same thermal history areas) use. In the Iberian Peninsula, fig. 1, four occupation areas have been defined: Atlantic Border, Mediterranean Border, Outback and Pyrenees.

In the Atlantic Border area *U. deningeri* appears in many caves: La Lucia (Quintanilla, Cantabria), Santa Isabel (Ranero, Vizcaya), Lezetxiki (Mondragón, Guipuzcoa). *U. spelaeus* appears in Eirós (Triacastela, Lugo), La Lucia, La Pasada (Guriezo, Cantabria), Lezetxiki, Arrikrutz (Oñate, Guipuzcoa), Ekain (Deba, Guipuzcoa), Troskaeta (Ataun, Guipuzcoa). In the Mediterranean Border there are not many caves with bear remains: Cova Bunica (Olopte, Girona) with *U. deningeri* and El Toll (Moiá, Barcelona) with *U. spelaeus*. In the Central Part of the Iberian Peninsula, the Sima de los Huesos (Atapuerca, Burgos), delivered many thousands of *U. deningeri* remains as well as hundreds of preneandertalian man bones and teeth, being dated (U-series and ESR), BISCHOFF *et al.* (1997), 320 ka old. El Reguerillo (Torrelaguna, Madrid) is the only important *U. spelaeus* locality in the area. In the Pyrenees: Coro Tracito (Tella, Huesca) represents the only high mountain locality. The Iberian Peninsula represents the species border: that means that palaeoenvironmental conditions were often near the species' stress limits. The cave bears moved south-wards only during climatic optimum periods appearing endogamy-dominated populations, as a response to minor palaeoenvironmental worsening in the European realm. Cave bears caverns are good sites for amino acid racemization analysis sampling because they are clean and that means that during the first and further taphonomical stages there are not too many possibilities of the arrival of foreign protein or amino acids. In

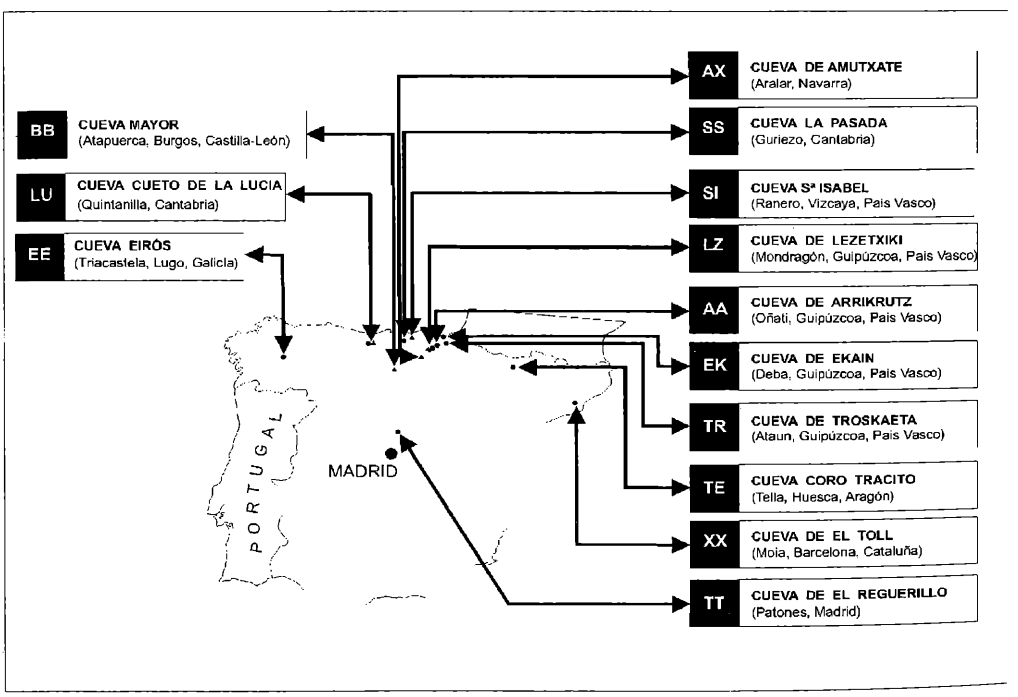

Figure 1: Geographical distribution of *Ursus deningeri* and *Ursus spelaeus* in the Iberian Peninsula with sampled localities situation.

Locality	Species	Latitude	Longitude	Altitude	Excavated
Sima Huesos, BB	*U. deningeri*	42° 20' 55,4" N	3° 0' 4,3" W	1022 m	Torres, 1976
Santa Isabel, SI	*U. deningeri*	43° 15' 27,1" N	3° 0' 4,4 W	300 m	Torres, 1982
Bunica, BU	*U. deningeri*	42° 23' 45,3" N	1° 48' 59,8" E	1200 m	Villalta, 1985
La Lucia, LU	*U. deningeri & U. spelaeus*	44° 54' 2,6" N	4° 33' 37,3" W	510 m	Torres, 1993-94
Eirós, EE	*U. spelaeus*	42° 15' 58,5" N	7° 11' 42,8" W	780 m	Torres, 1991-92
La Pasada, SS	*U. spelaeus*	44° 50' 22,7" N	1° 19' 15,8" W	460 m	Meijide-Fuentes, 1981
Arrikrutz, KK	*U. spelaeus*	43° 4' 11,6" N	3° 31' 50,1" W	453 m	Torres, 1975
Troskaeta, TR	*U. spelaeus*	43° 0' 7,3" N	2° 15' 1,4" W	580 m	Laborde-Elosegui, 1947; Torres, 1989-90
Coro Tracito, TE	*U. spelaeus*	42° 37' 10,2" N	5° 49' 5,2" W	1600 m	Torres-Canudo, 1995
El Toll, XX	*U. spelaeus*	42° 11' 5,7" N	2° 12' 31,4" E	760 m	Thomas-Villalta, 1957
El Reguerillo, TT	*U. spelaeus*	40° 53' 13,6" N	3° 26' 33,8" W	840 m	Torres, 1971-73

Table 1: Geographical situation of sampled localities.

fact, cave bears caverns are the best sites for amino acid racemization dating due to their thermal history did not show strong variations and the average temperature was moderately low. The Iberian Peninsula was never affected by general glaciation processes. River incision and hillside retreat were very strong: cave roof collapses closed ancient caves accesses. Local cave hydrogeological reactivations were very frequently the origin of mud beds deposition sealing bear remains. Amino acids (usually free amino acids) reached the cave deposits from soils and rhizosphere in general; some speleothems we have studied contained small amounts of aspartic acid.

In short: cave bears cavities are unusually clean sites with stable thermal history.

In spite of the aforementioned advantages the taphonomical process involved in a bear bone accumulation genesis seems to be markedly complex. With the only exception of scarce localities where the bear remains accumulation had an accidental cause (fall), their genesis was linked to a more or less wide time span of cave occupation by either bear females (with cubs and yearlings) or isolated males during winter hibernation periods. Starvation, ageing, parturition or disease linked deaths provided free dens for bear newcomer (male or female). After an unknown time span the cave would be reoccupied. Cave bears usually excavate a den on the muddy cave floor, and this "cave nest" can be described as a roughly hemispheric hole more than 1 cubic meter in volume. If the bear newcomer does not re-use the old den where the carcass of the former cave occupant is, it will dig a new one unearthing the old bear remains, destroying any pre-existing stratigraphy and scattering any previously articulated skeletal remains. Anyway a change in a previous diagenetical conditions will be produced. In any case these diagenetical processes will not decisively act on the finally reached average racemization ratio but will "retouch" the process in such a way that there will be higher racemization values dispersion. This to some degree can be explained as a response to a "micro-environmental mosaic" that overlaps with the inter-sample racemization ratio differences effect which can be explained in merely chronostratigraphic terms: different periods of cave occupation by cave bear individuals.

2. Materials and methods

We have analyzed samples of *U. deningeri* (Sima de los Huesos – BB, Coba Bunica – BU, La Lucia cave – LU and Santa Isabel Cave – SI) and *U. spelaeus* (El Reguerillo cave – TT, La Lucia cave – LU, La Pasada cave – SS, El Toll cave – XX, Troskaeta cave – TR, Eirós cave – EE and Coro Tracito cave – TE) see table 1. We have selected five canines or third upper incisors from each locality because their crown conic shape protects efficiently dentine from contamination. When it has been possible we have chosen perfect specimens of intermediate age ones; not very old with strongly worn enamel and open pulp cavity, and not very young, with open apex root or and thin dentine layers. In any case we have tried to sample "sealed" teeth.

50 mg of powdered dentine samples were obtained from the innermost part of the crown via drilling the tooth with a dental diamond drill. Powder from the outer part of the root, up to the limit of 1 mm deep was rejected, that means that cement layers have never been sampled. This process produced an unavoidable slight sample heating.

Because former results were not consistent when free and bonded aspartic acid racemization was analyzed

as a whole, before performing hydrolysis and amino acid derivatization, the samples were treated to eliminate free amino acids. With this process we aimed to remove foreign amino acids and to obtain a homogeneous "collagen extract" For this purpose we have used a modification of the method proposed by MARZIN (1990). The powder sample, 50 mg of dentine, was dissolved in 1 ml of 2N hydrochloric acid and sonicated. Following the addition of 5 ml of PBS buffer, the sample was dialyzed at 3500 Dalton (Spectra/Por mnco 3500 membrane) for a period of 20 h in a buffered solution with magnetic stirring at room temperature. Glassware used in the analyses (except Pasteur pipettes) were cleaned by baking in an oven at 500°C for about 2 hr. Eppendorf plastic micro test tubes, plastic micropipette tips and Pasteur pipettes were new from factory. Teflon liners and septa were thoroughly washed with petroleum ether, acetone and rinsed three times with ultraclean water. All the water used in the analysis was Milli-Q quality from Millipore. Concentrated hydrofluoric and hydrochloric acids and trifluoroacetic acid anhydride were Merck analytical grade. Thionyl chloride was purchased from Fluka AG. Isopropyl alcohol and n-hexane were Merck HPLC grade, and dichloromethane was Merck spectroscopy grade.

Hydrolysis was carried out in a mixture of 12 N hydrochloric acid (2.9 m l/mg) and 6 N hydrochloric acid (100 ml), in test tubes with Teflon lined screw caps closed under nitrogen atmosphere, in a heating block at 100°C for 20 hours. Samples were transferred to conical 1.5 ml Eppendorf plastic micro test tubes with caps, concentrated hydrofluoric acid (1.25 (ml/mg of sample) was added, and the tubes were mixed with a mechanical Vortex shaker, and centrifuged for 4 minutes in an Eppendorf centrifuge. The supernatant was transferred into new 1.5 ml Eppendorf micro test tubes, frozen in liquid nitrogen, and vacuum dried in a plastic desiccator. Samples were redisolved with 80 ml distilled water, mixed in the Vortex shaker, centrifuged for a few seconds to get all droplets down and transferred into 2 ml glass vials, with screw caps and Teflon-lined septa. Water was evaporated at vacuum from the cap-covered, not tight closed, vials in the plastic desiccator.

The first derivatization step of the amino acids was the esterification with 250 ml of 3 M thionyl chloride in isopropanol. The vials were tightly closed under nitrogen and let react on the heating block at 100°C for just 1 hour. Afterwards, the vials were opened but not uncovered in a hood, and vacuum dried in a plastic desiccator just to dryness, not longer. The second derivatization step was the N-trifluoroacetylation with 150 ml of trifluoroacetic acid anhydride (25% in dichloromethane). The vials were tightly closed under nitrogen and heated at 100°C for just 5 minutes on the heating block. Later were allowed to cool, and opened in a hood, where the dichloromethane solvent, and the unreacted trifluoroacetic acid anhydride, were evaporated under a gentle flow of nitrogen. Later the samples were dissolved in 125 ml of n-hexane, shaken in the Vortex, and most of the n-hexane was evaporated in a stream of nitrogen to a final volume of 15-25 ml and transferred to 150 ml injection vials.

0.2 ml of recent bear sample (*U. spelaeus*) or 2 ml of old bear (*U. deningeri*) were injected into a Hewlett-Packard 5890 gas chromatograph. The injection port was kept at 215°C and set for splitless mode for the first 75 s, at the beginning of which the sample was injected, and later set to split mode. We used helium as the carrier gas, at a column head pressure of 5.8 psi, and a Chirasil-Val fused silica column (0.39 mm x 0.25 mm x 25 m) from Chrompack. The gradients we used were as follows: 50°C (1 min.), heat at 40°C/min. to 115°C, remain at 115°C for 12 min., heat at 3°C/min. to 190°C, remain at 190°C for 10 min., cool down to 50°C and remain at this temperature between runs (80°C if the time between runs is longer, typically overnight). The detector was a NPD set at 300°C. Integration of the peak areas was carried out using the HP's PEAK96 integration program from Hewlett-Packard that runs on a PC computer. The sensitivity limits of the method could be fixed according to the method induced racemization (0,00-0,03 depending on the amino acid considered) and the minimum amino acid concentration detectable into the samples. As a laboratory routine D/L-valine, D/L-alanine, D-*Allo*isoleucine/L-isoleucine, D/L-proline, D/L- aspartic acid, L-hydroxyproline, D/L-phenilalanine and D/L-glutamic acid peaks were identified.

3. Discussion and conclusions

Analyses results appear in a table, tab. 2 and in a graph, fig. 2. From both elemental statistics table and histogram we can define a clearly identifiable *Ursus deningeri* aminozone on the Iberian Peninsula, with aspartic acid racemization ratios over 0,30 and average values close to 0,32. This fact is especially evident when comparing the mean values of racemization of the aspartic acid of *Ursus spelaeus* and *Ursus deningeri* from La Lucia cave (LU), where the mean values of racemization for aspartic acid are, 0,13 and 0,33, respectively. We conclude that materials classified purely on paleontological grounds as *Ursus deningeri* have a chronostratigraphic value and therefore cannot constitute a case of polytypism within a single species. In *Ursus spelaeus* localities, most of the racemization ratios concentrate below 0,10.

The *Ursus spelaeus* aspartic acid racemization ratios define a broad aminozone in which two subaminozones may be distinguished: one with two higher racemization ratio populations (El Reguerillo and Arrikrutz) and the other, El Toll, La Lucia, Eirós, Troskaeta, Coro Tracito,

Species	Locality	CMAT	N	Mean	Std	CV (%)
U. spelaeus	Eirós (EE)	10,0–12,5	5	0,0680	0,0110	11,07
U. spelaeus	Coro Tracito (TE)	7,0–10,0	5	0,0740	0,0251	33,92
U. spelaeus	Troskaeta (TR)	12,5–15,0	4	0,0850	0,0129	15,18
U. spelaeus	El Toll (XX)	12,5–15,0	5	0,0980	0,0084	8,57
U. spelaeus	La Pasada (SS)	12,5–15,0	4	0,1025	0,0377	36,78
U. spelaeus	La Lucia (LU)	12,5–15,0	5	0,1340	0,0336	25,07
U. spelaeus	Arrikrutz (AA)	10,0–12,5	6	0,1867	0,0455	24,37
U. spelaeus	Reguerillo (TT)	10,0–12,5	9	0,1985	0,0651	32,79
U. deningeri	Bunica (BU)	7,0–10,0	2	0,2750	0,0353	12,84
U. deningeri	Sima los Huesos (BB)	10,0–12,5	15	0,3200	0,0571	17,84
U. deningeri	La Lucia (LU)	12,5–15,0	3	0,3300	0,0265	8,03
U. deningeri	Santa Isabel (SI)	10,0–12,5	7	0,3457	0,0898	25,98

Table 2: Elemental statistics of aspartic acid racemization ratios from the different *U. deningeri* and *U. spelaeus* sampled localities. n = number of analyses; m = mean; s = standard deviation; CV= coefficient of variation (s/m %); CMAT = Current Mean Annual Temperature range.

and La Pasada, with very low aspartic acid racemization ratios.

The Arrikrutz (AA) and El Reguerillo (TT) caves have higher average aspartic acid racemization values: 0,19 and constitute a distinctive *U. spelaeus* subaminozone. Big sized true cave bear from El Reguerillo and Arrikrutz caves have the highest aspartic acid racemization ratios. Both "European normal sized populations" could be interpreted as the former true cave bear inhabitants.

Consequently, it is possible to establish a subaminozone of aspartic acid racemization, which would include most of the Iberian locations of *Ursus spelaeus.*

The material from the Coro Tracito site (TE) has a low mean value of racemization of aspartic acid (0,08). We consider that this lower value may be due to the high altitude (1600 m) of this site compared with the others, which would decrease the rate of racemization due to the lower CMAT of this locality. This site might therefore be older than the others in this group. The dentine from cave bears with short and broad paws (only in some localities) have the lowest aspartic acid racemization ratios at all. They existed alongside with the extant Iberian brown bear, in some cases confined to less favorable craggy areas. Short pawed bears from *Ursus spelaeus parvilatipedis* TORRES group seem to

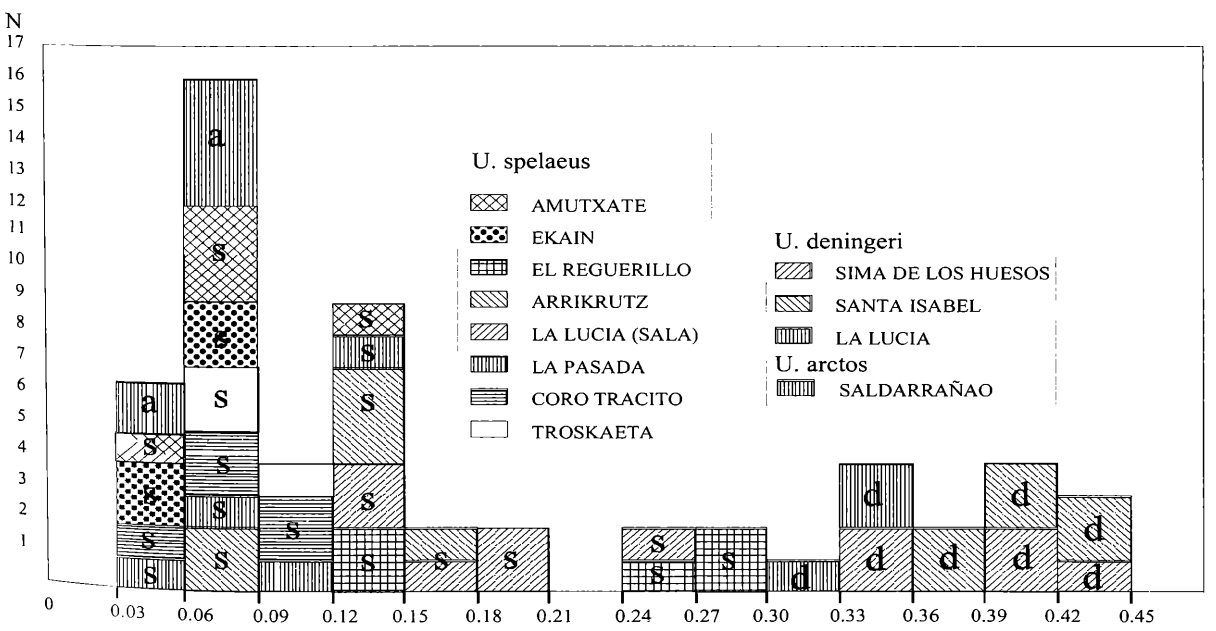

Figure 2: Histogram of aspartic acid racemization ratios from diferent localities and species.

compose a relative homogenous group which inhabited the Iberian Peninsula at the end of the last maximum glacial. One of their representatives, Eirós cave has been ^{14}C dated ca. 27 ka BP, cf. GRANDAL d'ANGLADE & VIDAL ROMANÍ (1997).

Big pawed cave bears from el Reguerillo (TT) and Arrikrutz (AA) must be older than the La Lucia (LU) cave *U. spelaeus*, ca. 85 ka BP, according to U/Th-dating from a flowstone sealing the deposit and a stalactite scattered into the bear bearing sediment. Aspartic acid racemization analysis from cave bear species dentine collagen has been proved to be proxy for the establishing of the Iberian cave bear amino stratigraphy. Materials classified purely on palaeontological grounds as *Ursus deningeri* have a chronostratigraphical value and therefore cannot constitute a case of polytypism within a single species. In fact the Sima de los Huesos bear remains have been dated using the U-series and ESR methods at 320 ka BP, cf. BISCHOFF et al. (1997).

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