Evidence for Reproductive Isolation between Cave Bear Populations

Michael Hofreiter, Gernot Rabeder, Viviane Jaenicke-Després, Gerhard Withalm, Doris Nagel, Maja Paunovic, Gordana Jambrěsić, and Svante Pääbo

Supplemental Results

Dating

Cave bear remains from three caves (Figures 1A and 1B) were dated. Gamssulzen Cave (1300 m altitude) and Ramesch Cave (1960 m altitude) are situated approximately 10 km apart in the Austrian Alps (Figure 1B) and are not separated by any obvious physical barriers, while Vindija Cave (379 m altitude) is in Croatia, app. 200 km from the other two caves (Figure 1A). Carbon dates were determined for nine cave bear remains from Ramesch Cave, seven cave bear remains from Gamssulzen Cave, and ten cave bear remains from Vindija Cave in Croatia (Table S1). In combination with published carbon- and uranium-series dates [S3, S12, S13] this allows the occupation times of the three caves to be estimated. Gamssulzen Cave was inhabited by cave bears from approximately 47,000 years before present (BP) to approximately 26,000 years BP, while Ramesch Cave was inhabited continuously from approximately 64,000 years BP to approximately 31,000 years BP, as well as during an earlier period, from approximately 150,000 to approximately 120,000 years BP. Vindija Cave had the longest history of cave bear occupation, from at least 50,000 years BP to approximately 23,000 years BP, as well as during an earlier period from at least 130,000 years BP to approximately 70,000 years BP [S13]. As calibration of radiocarbon dates is currently not possible beyond 24,000 cal years BP [S14], we used noncalibrated radiocarbon dates for estimating the occupation times of the caves. However, it is not known in which direction radiocarbon ages deviate from true age beyond 35,000 radiocarbon years BP [S14]. If at older ages radiocarbon dates were older than the chronological age, this would reduce the time of contemporaneous occupation of Ramesch and Gamssulzen Caves. As most data indicate that radiocarbon dates are younger than the chronological age up to the limit of radiocarbon dating [S14], and a number of uranium series dates from Ramesch Cave also range from 32,000 to >50,000 years BP (Figure 2), 15,000 years seems to be a good estimate for the time span of contemporaneous occupation of Ramesch and Gamssulzen Caves.

Table S1. Radiocarbon Dates for the Cave Bear Samples from Gamssulzen, Ramesch, and Vindija Caves

Radiocarbon Dates (Uncorrected)	
Gamssulzen Cave	**Age**
GS31 (157659)	32,190 ± 330
GS32 (157660)	32,060 ± 320
GS33 (157661)	37,310 ± 580
GS34 (157662)	44,400 ± 1380
GS35 (157663)	45,410 ± 1560
GS36 (157664)	41,060 ± 920
GS37 (157665)	47,300 ± 1970
Ramesch Cave	**Age**
R3451 (157666)	>50,000
R3452 (157667)	43,700 ± 1270
R3453 (157668)	>50,000
R3454 (157669)	47,600 ± 2060
R3455 (157670)	31,140 ± 310
R3456 (157671)	>50,000
R3491 (157672)	>50,000
R291 (143241)	49,520 ± 1600
R293 (143242)	43,610 ± 800
Vindija Cave	**Age**
VG1573 (143247)	38,770 ± 500
VG1574 (156095)	42,620 ± 1150
VG1578 (156096)	43,230 ± 1200
VG1584 (156087)	45,210 ± 1220
VG1585 (143248)	31,990 ± 300
VG1587 (156098)	48,990 ± 1950
VG1595 (156099)	41,100 ± 930
VG1599 (156100)	23,780 ± 120
VH2432 (156101)	42,660 ± 900
VK8035 (156103)	Insufficient collagen
VG006	33,335 ± 145

Morphological Analyses

Overall we tested 30 morphological parameters, 20 concerning metric measurements of teeth and metapodial bones and 10 concerning the length to breadth proportion of metapodial bones (Table S2). The Ramesch and Gamssulzen bears differ both for metric measurements (only teeth) and in the metapodial bone proportions. Similarly, the Vindija and Ramesch bears differ also for metric measurements (both metapodial bones and teeth) and in the metapodial bone proportions. Contrary to that, the Vindija and Gamssulzen bears differ only for metric comparisons, but not in the metapodial bone proportions. Thus, the Vindija bears were larger overall than the Gamssulzen bears but did not differ in their body proportions. The metric differences are likely to be determined by the different environment around the two caves. The fact that the Ramesch bears had smaller teeth than the Gamssulzen bears and at the same time metapodial bones of the same length but more slender than those of the Gamssulzen bears shows that these two populations differed in their body proportions. Moreover, overall, the Gamssulzen and Ramesch bears are significantly different for more parameters than the Gamssulzen and Vindija bears (Table S2), despite the former two caves both being geographically much closer (<10 km versus approximately 200 km) and differing less in altitude (700 m difference versus 900 m difference) than the latter two. All tests were done without correction for multiple testing. If a Bonferroni correction is applied, the number of comparisons that are significantly different reduces for some metric measurements for both the Vindija Gamssulzen comparison (8 versus 12) and the Vindija Ramesch comparison (15 versus 19), but neither for the Gamssulzen Ramesch comparison nor for any of the comparisons for metapodial bone proportions. Overall, the Vindija and Ramesch bears differ for 24 of 30 parameters after Bonferroni correction, the Vindija and Gamssulzen bears for 8 of 30, and the Gamssulzen and Ramesch bears for 19 of 30.

We also compared the different stratigraphical layers within Ramesch Cave, which correspond to different ages during which the bones were deposited [S3] for the above tested parameters. We did not find a statistically significant difference between layers for any of the parameters.

mtDNA Amplification

For two of the samples, only two of the fragments could reproducibly be amplified (Figure S3). Although a sequence from a single amplification was available for the third fragment from one of these samples, it was excluded from the analyses to avoid errors due to nucleotide misincorporations [S8, S15].

Likelihood of Gene Flow

Ramesch Cave contains the remains of a minimum number of 182 individuals and Gamssulzen Cave contains the remains of a minimum number of 81 individuals. As a cave bear skeleton contains approximately 290 bones, the two caves originally contained about 53,000 and 23,500 bones, respectively. As the bones in the two caves were disarticulated, as it is typical for cave bear caves [S16],

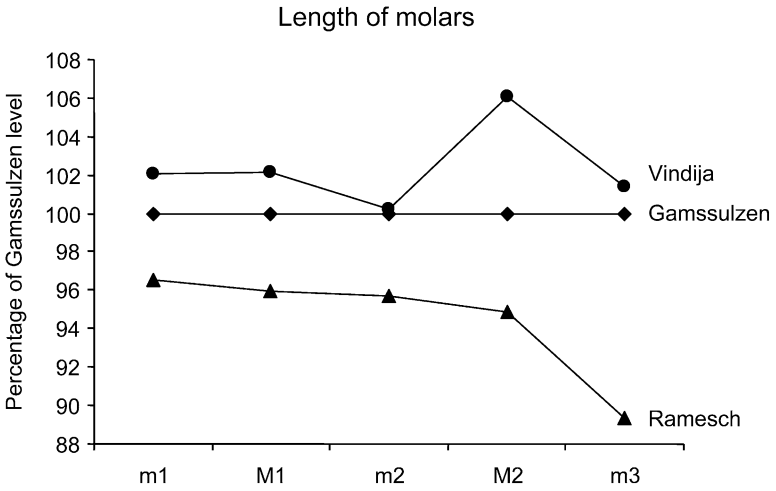

Figure S1. Average Length of Five Molars in Relation to the Values from Gamssulzen Cave (set at 100%)

m1, first lower molar; M1, first upper molar; m2, second lower molar; M2, second upper molar; m3, third lower molar. Values for teeth from Vindija Cave are shown by dots, those from Gamssulzen Cave by diamonds, and those from Ramesch Cave by triangles.

the likelihood for any bone to carry one or the other sequence type depends only on the proportion of the two sequence types in the original population. Thus, the power to reject a panmictic population depends only on the number of bone samples investigated. As the sampling of the two caves represents two independent experiments, the overall likelihood to discover only sequences of one type in one cave and only sequences of the other type in the second cave is $p^{n1} \times (1 - p)^{n2} + p^{n2} \times (1 - p)^{n1}$. In our case, this is $(5/12)^5 \times (7/12)^7 + (5/12)^7 \times (7/12)^5 = 0.044\%$. However, a conservative estimate can be done by using only samples that cannot originate from the same individual or from close relatives such as mother-offspring or sibling pairs. This possibility can be excluded for three samples from Ramesch and four from Gamssulzen Cave, respectively, if nonoverlapping carbon dates at two standard deviations are used. Using these numbers, the probability to recover only one type of mtDNA sequences from Ramesch Cave and only the other type of mtDNA sequences from Gamssulzen Cave is still only 1.5% if the bears from the two caves belonged to a panmictic population.

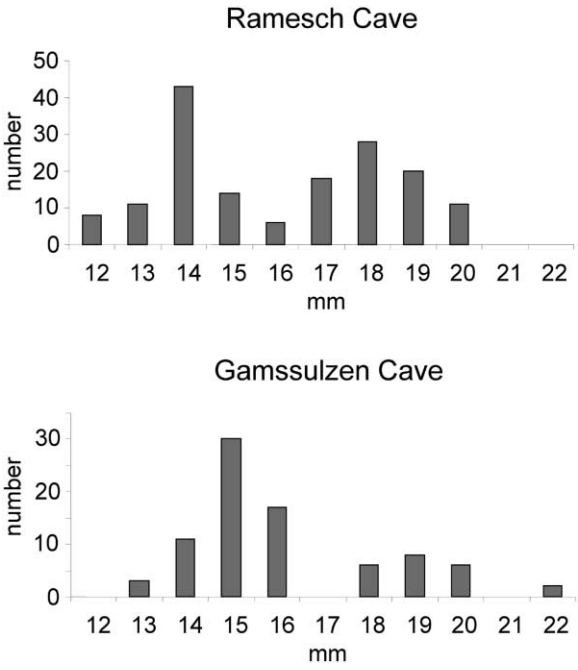

Figure S2. Breadth of the Canines from Ramesch and Gamssulzen Caves

For both caves, a bimodal distribution is found, with the smaller ones representing females, the larger ones males. Note that the proportion of females was larger in Gamssulzen Cave than in Ramesch Cave, contradicting the suggestion [S17] that a higher proportion of females lead to the smaller size of high Alpine cave bears.

Supplemental Experimental Procedures

Dating

Samples that yielded DNA were ^{14}C dated by tandem accelerator mass spectroscopy (AMS) at BETA Analytic, Inc. (Miami, Florida). In all analyses we used noncalibrated carbon dates as the latest radiocarbon calibration curve (INTCAL 98) extends to only 15,585 cal BP [S1], and calibration of radiocarbon dates is not yet possible beyond 24,000 cal B.P. [S2]. Additional dates were taken from the literature [S3].

Bone and Teeth Measurements

Bone and teeth measurements were done to the nearest tenth of a millimeter. Width and depth of teeth were measured because these measures do not change during the adult life in mammals, whereas the length of teeth is altered by wear. For measurements of the metapodial bones, only bones with fully ossified epiphyses were used to exclude juvenile animals. Thickness of the metapodial bones was calculated from the length to breadth ratio and standardized as described [S4].

DNA Extraction

Bone or teeth were ground with mortar and pistil. 10 ml extraction buffer containing 0.45M EDTA (pH 8), 0.5% N-Lauroylsarcosine, 1% Polyvinylpyrolidone, 50 mM DTT, 2.5 mM PTB [S5, S6], and 0.25 mg/ml Proteinase-K were added to 200 mg^{-1} g of bone powder and incubated for 16 hr at 37°C under rotation. The remaining bone powder was collected by centrifugation and only the supernatant was used for further processing. DNA was purified by binding to silica. 40 ml of L2 buffer (5.5 M Guanidinium-isothiocyanate, 25 mM NaCl, 100 mM Tris [pH 8]) and 50 μl of silica suspension were added to 10 ml supernatant and incubated for approximately 30 min. The silica pellet was collected by brief centrifugation, the supernatant discarded, and the DNA was further purified as described [S6] except that the silica pellet was washed only once with NewWash. The final volume of the extract was 100 μl, of which 5 μl were used for a single PCR reaction. PCRs contained 1.25 U AmpliTaq Gold (Perkin Elmer, USA), 1× AmpliTaq Gold buffer, a final concentration of 250 μM for each dNTP, 250 nM for each primer, and 2.5 mM $MgCl_2$ in a final volume of 40 μl. Primary amplifications were done on a MJ Thermo cycler with a 3 min activation step at 94°C, followed by 60 cycles at 93°C for 30 s, 47°C–50°C for 60 s, and 72°C for 45

Table S2. Results from Pairwise Comparisons between the Three Caves for 30 Morphological Parameters

Comparison	Vin—Ram	Vin—Gam	Gam—Ram
Metric parameters			
m1 length	$p < 0.01$	ns	$p < 0.01$
m1 breadth	$p < 0.01$	$p < 0.01$	$p < 0.01$
m2 length	$p < 0.01$	$p < 0.01$	$p < 0.01$
m2 breadth	$p < 0.01$	$p < 0.01$	$p < 0.01$
m3 length	$p < 0.01$	ns	$p < 0.01$
m3 breadth	$p < 0.01$	$p < 0.01$	$p < 0.01$
M1 length	$p < 0.01$	ns	$p < 0.01$
M1 breadth	$p < 0.01$	ns	$p < 0.01$
M2 length	$p < 0.01$	$p < 0.01$	$p < 0.01$
M2breadth	$p < 0.01$	ns	$p < 0.01$
mc1 length	$p < 0.01$	ns	ns
mc2 length	$p < 0.01$	$p < 0.01$	ns
mc3 length	$p < 0.01$	ns	ns
mc4 length	$p < 0.01$	$p < 0.01$	ns
mc5 length	$p < 0.01$	$p < 0.01$	ns
mt1 length	$p < 0.01$	$p < 0.01$	ns
mt2 length	$p < 0.01$	$p < 0.01$	ns
mt3 length	$p < 0.01$	ns	ns
mt4 length	$p < 0.01$	$p < 0.01$	ns
mt5 length	ns	$p < 0.01$	ns
Summary for Metric Parameters	19	12	10
Thickness			
mc1	ns	ns	ns
mc2	$p < 0.01$	ns	$p < 0.01$
mc3	$p < 0.01$	ns	$p < 0.01$
mc4	$p < 0.01$	ns	$p < 0.01$
mc5	$p < 0.01$	ns	$p < 0.01$
mt1	$p < 0.01$	ns	$p < 0.01$
mt2	$p < 0.01$	ns	$p < 0.01$
mt3	$p < 0.01$	ns	$p < 0.01$
mt4	$p < 0.01$	ns	$p < 0.01$
mt5	$p < 0.01$	ns	$p < 0.01$
Summary for Thickness	9	0	9
Overall	28	12	19

The distributions were compared using a two-tailed t-test assuming unequal variance. m1, lower first molar; m2, lower second molar; m3, lower third molar; M1, upper first molar; M2, upper second molar; mc1–5, metacarpals 1–5; mt 1–5, metatarsals 1–5. Vin, Vindija Cave; Gam, Gamssulzen Cave; Ram, Ramesch Cave, ns, non significant.

s. PCR products were isolated from 2.8% agarose gels and melted in 100 μl double-distilled water. 5 μl of the melted product was used for reamplifications for 30 cycles under the PCR conditions described, except that the activation step was prolonged to 7 min, and the annealing temperature was always 52°C. Reamplification products were cloned using the Topo TA cloning kit (Invitrogen, the Netherlands). In cases when no primer dimers were observed in the primary amplification, the product was cloned directly. Primers used were: CB2670a (1F), 5′-CTATTTAAACTATTCCCTGGTACATAC-3′; CBH45 (1R), 5′-GGACATACTATGATGGTACAGTACAT-3′; CBL130 (2F), 5′-CTATGTATATCGTGCATTGGC-3′; CBH177a (2R), 5′-AAACT TTCGAAATGTAGGTCCTC-3′; CBL164 (3F), 5′-GCATATAAGCATGT ACATATTATGC-3′; CBH221 (3R), 5′-CGGACTAAG TGAAATACAT GCT-3′.

Clones were sequenced on an ABI 3700 capillary sequencer from purified colony PCRs [S7]. Purification of colony PCR products was done on a Biorobot 9600 (Qiagen) using the Qiagen PCR Purification Kit (Qiagen). For each segment, a minimum of six clones, three from each of two independent amplifications, was sequenced using M13 universal primers. If all clones from the first amplification consistently differed from all clones from the second amplification at one or more positions, a third amplification was done from the extract to determine which sequence was reproducible [S8]. Mock extractions without sample and PCR blanks were performed to monitor contamination.

Sequence Analysis

Altogether, 134 bp of the cave bear mtDNA control region were amplified in two overlapping (46 and 56 bp, respectively, excluding primers) and one nonoverlapping piece (44 bp, excluding primers). The fragments covered 18 out of a total of 23 variable positions detected earlier [S9, S10]. The sequences were aligned by eye to the most common sequence from Vindija Cave. A median joining network was reconstructed using the program Network [S11].

Likelihood of Gene Flow

If a panmictic population is assumed, the likelihood to find one of two types of sequences in k of n analyzed bones is described by the binomial distribution, with p being the frequency of one sequence type in the population and $1 - p$ being the frequency of the other sequence type. If all samples investigated belong to only one type, the binomial distribution simplifies to p^n (or $(1 - p)^n$ depending which type of sequence was found).

Supplemental References

S1. Stuiver, M., Reimer, P., Bard, E., Beck, J., Burr, G., Hughen, K., Kromer, B., McCormac, G., van der Pflicht, J., and Spurk, M. (1998). INTCAL98 radiocarbon age calibration, 24,000–0 cal BP. Radiocarbon *40*, 1041–1083.

S2. Kitagawa, H., and van der Pflicht, J. (2000). Atmospheric radiocarbon calibration beyond 11,900 cal BP from Lake Suigetsu laminated sediments. Radiocarbon *42*, 369–380.

S3. Rabeder, G. (1999). Die Evolution des Höhlenbärgebisses (Wien: Verlag der Österreichischen Akademie der Wissenschaften).

S4. Withalm, G. (2001). Die Evolution der Metapodien in der Höhlenbär-Gruppe (Ursidae, Mammalia). Beiträge zur Paläontologie *26*, 169–249.

S5. Vasan, S., Zhang, X., Kapurniotu, A., Bernhagen, J., Teichberg, S., Basgen, J., Wagle, D., Shih, D., Terlecky, I., Bucala, R., et al. (1996). An agent cleaving glucose-derived protein crosslinks in vitro and in vivo. Nature *382*, 275–278.

S6. Poinar, H.N., Hofreiter, M., Spaulding, W.G., Martin, P.S., Stankiewicz, B.A., Bland, H., Evershed, R.P., Possnert, G., and Pääbo, S. (1998). Molecular coproscopy: dung and diet of the extinct ground sloth Nothrotheriops shastensis. Science *281*, 402–406.

S7. Kilger, C., Krings, M., Poinar, H., and Pääbo, S. (1997). "Colony sequencing": direct sequencing of plasmid DNA from bacterial colonies. Biotechniques *22*, 412–414, 416, 418.

S8. Hofreiter, M., Jaenicke, V., Serre, D., Haeseler, Av., and Pääbo, S. (2001). DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. Nucleic Acids Res. *29*, 4793–4799.

S9. Hofreiter, M., Capelli, C., Krings, M., Waits, L., Conard, N., Munzel, S., Rabeder, G., Nagel, D., Paunovic, M., Jambresic, G., et al. (2002). Ancient DNA analyses reveal high mitochondrial DNA sequence diversity and parallel morphological evolution of late Pleistocene cave bears. Mol. Biol. Evol. *19*, 1244–1250.

S10. Loreille, O., Orlando, L., Patou-Mathis, M., Philippe, M., Taberlet, P., and Hänni, C. (2001). Ancient DNA analysis reveals divergence of the cave bear, Ursus spelaeus, and brown bear, Ursus arctos, lineages. Curr. Biol. *11*, 200–203.

S11. Bandelt, H.J., Forster, P., and Rohl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. *16*, 37–48.

S12. Doeppes, D., and Rabeder, G. eds. (1997). Pliozäne and pleistozäne Faunen Oesterreichs, Volume 10 (Wien: Oesterreichische Akademie der Wissenschaften).

S13. Wild, E., Paunovic, M., Rabeder, G., Steffan, I., and Steier,

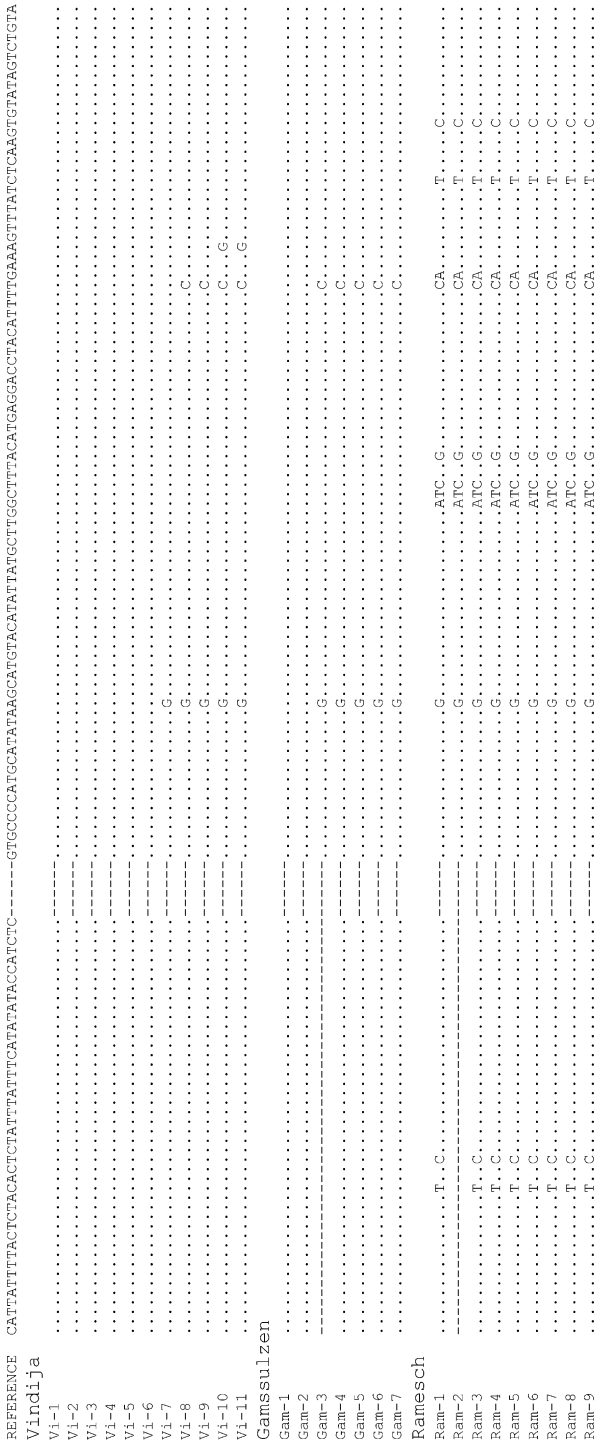

Figure S3. The mtDNA Sequences Recovered from the Three Caves

Dots indicate identity. The six dashes in all sequences represent a gap of about 85 bp that separates the two nonoverlapping fragments analyzed. For two samples, the first fragment could not be amplified twice. This is indicated by dashes.

P. (2001). Age determination of fossil bones from the Vindija Neanderthal site in Croatia. Radiocarbon *43*, 1021–1028.

S14. van der Pflicht, J. (2002). Calibration of the 14C time scale: towards the complete dating range. Netherlands Journal of Geoscience *81*, 85–96.

S15. Gilbert, M.T., Hansen, A.J., Willerslev, E., Rudbeck, L., Barnes, I., Lynnerup, N., and Cooper, A. (2003). Characterization of genetic miscoding lesions caused by postmortem damage. Am. J. Hum. Genet. *72*, 48–61.

S16. Kurten, B. (1976). The Cave Bear Story (New York: Columbia University Press).

S17. Reisinger, C., and Hohenegger, J. (1998). Sexual dimorphism in limb bones of Late Pleistocene cave bears (*Ursus spelaeus*, Carnivora, Mammalia) from three caves in Eastern Alps (Austria and Italy). Bolletino del la Societa Paleontologica Italiana *37*, 99–116.

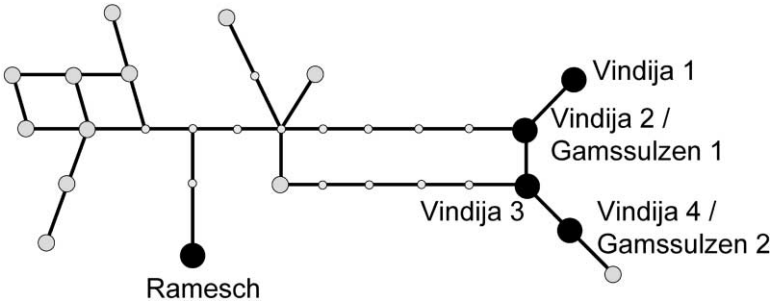

Figure S4. Median Joining Network for 17 Different Cave Bear mtDNA Sequences

Large black dots represent mtDNA sequences from Ramesch, Gamssulzen, and Vindija, large gray dots mtDNA sequences observed elsewhere [S9, S10], and small gray dots inferred sequences. The network was reconstructed using the program Network [S11].