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Ancient DNA and dating of cave bear remains from Niedźwiedzia Cave suggest early appearance of *Ursus ingressus* in Sudetes

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ABSTRACT

The migration of *Ursus ingressus* from Southern to Western Europe, where it replaced native forms of *Ursus spelaeus*, is well documented. However, its occurrence in Central and Eastern European countries is unexplored. We have characterized the cave bear population inhabiting the Sudetes Mountains during the Late Pleistocene. DNA analyses were performed on 39 cave bear specimens from the Niedźwiedzia Cave. Ten successfully yielded the 254-bp sequences of an mtDNA control region. Phylogenetic analyses allowed assignment of nine samples to the *U. ingressus* haplogroup and one to brown bear (*Ursus arctos*). The mtDNA haplotypes of *U. ingressus* formed a single cluster and differed from haplotypes obtained for cave bears from other excavation sites in Europe. Uranium–thorium and radiocarbon dating of selected samples has shown that *U. ingressus* appeared in the Sudetes region at least 80,000 years ago. This finding may suggest that the appearance of *U. ingressus* in this area resulted from migration that occurred earlier than the colonization of the Alps and Swabian Jura. It is possible that migration of *U. ingressus* proceeded along the Carpathian and Sudetes arc.

1. Introduction

It is widely accepted that two main forms of cave bear, *Ursus ingressus* and *Ursus spelaeus*, inhabited Europe during the Late Pleistocene (Rabeder, 1995; Rabeder et al., 2000, 2004a, 2004b; Rabeder and Hofreiter, 2004; Hofreiter et al., 2004b) (Fig. 1). However, the taxonomical position of these forms is still under debate: they are considered either as separate species (Rabeder, 1995; Rabeder and Hofreiter, 2004; Rabeder et al., 2004a, 2004b; Hofreiter et al., 2004b) or as *U. s. spelaeus* and *U. s. ingressus*, subspecies of *U. spelaeus* (Baryshnikov and Puzachenko, 2011). *U. spelaeus* has been further divided into 'classic' *U. s. spelaeus* and two high-Alpine forms with reduced body size, *U. s. eremus* and *U. s. ladinicus* (Rabeder et al., 2004b, 2008). *U. spelaeus* remains have been found in Spain, France, Germany, Belgium, Italy, and Austria, while *U. ingressus* inhabited mostly Eastern Europe and has been discovered in Romania, Slovenia, Ukraine, Czech Republic, Slovakia, and Greece but also in Switzerland, Austria, and Germany. Divergence of these two forms took place 173–414 ka (Knapp et al., 2009). The place of *U. ingressus* origin remains unknown: however, the basal position of haplotypes from the Romanian site Peştera cu Oase in phylogenetic trees suggests Southeastern Europe (Baca et al., 2012). It has been proposed that the westward migration of *U. ingressus* started ca. 60,000 years ago (Rabeder and Hofreiter, 2004; Münzel et al., 2011). The presence of *U. ingressus* was genetically confirmed in several sites in Austria (Gamssulzen, Nixloch, and Herdengel caves), and its first appearance has been dated to ca. 50,000 years ago (Rabeder and Hofreiter, 2004; Hofreiter et al., 2004a, 2004b). *U. ingressus* cohabited with Alpine forms of *U. spelaeus* for some time and ultimately replaced it. Further migration of *U. ingressus* to the Swabian Jura ca. 32,000

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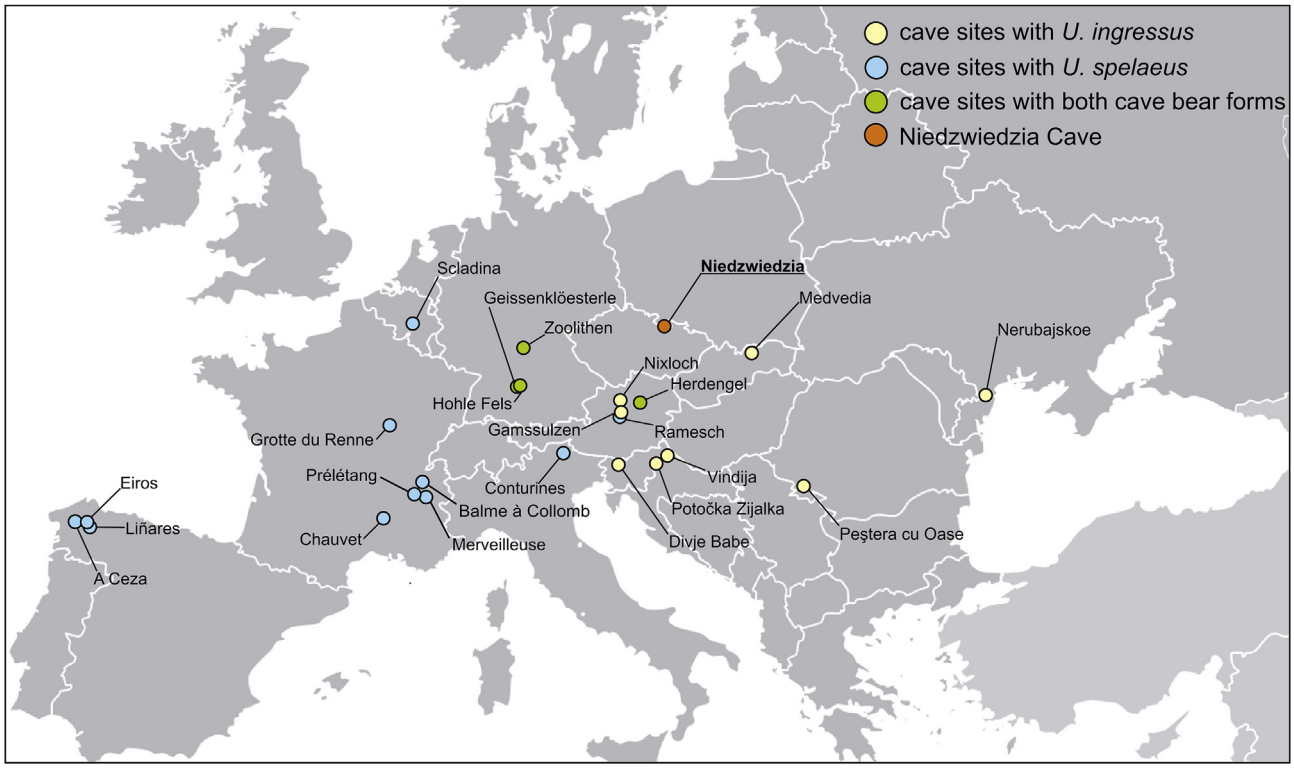

Fig. 1. Distribution of European excavation sites where genetically confirmed *U. spelaeus* and *U. ingressus* remains were found.

years ago probably also resulted in the final replacement of *U. spelaeus* ca. 28,000 years ago (Hofreiter et al., 2007; Münzel et al., 2011).

We previously reported two aDNA sequences of this species from Niedźwiedzia Cave in Poland, moving its range northwards (Baca et al., 2012). The taxonomical affiliation of these samples was confirmed by phylogenetic and morphometric analyses. One sample was found to be over 49,000 years old, indicating an earlier-than-expected migration of *U. ingressus* into the Sudetes region. To verify this interesting hypothesis and to assess the genetic diversity of cave bears from this region, we have carried out aDNA analyses on a larger sample and applied uranium–thorium dating of bone collagen to obtain the precise age of the studied samples.

2. Materials and methods

2.1. Excavation site and samples

Niedźwiedzia Cave (Bear Cave) (50° 14′ 03″ N; 016° 50′ 03″ E) is located in the village of Kletno, Stronie Śląskie commune, in the Śnieżnik Massif of the Sudetes (Figs. 1 and 2). It was discovered in 1967 and has been systematically explored since (Wiszniowska, 1976, 1978, 1989; Wiszniowska et al., 1996; Bieroński et al., 2009). Its deposits including animal remains were dated from the upper Weichselian (Vistulian) glacial stage (>50,000–12,000 BP) to the Holocene in the top parts of some profiles. The faunal assemblage from the cave consists of tens of thousands of vertebrate remains, and those of the cave bear constitute 70%–90% of all fossils collected. Radiocarbon dating of the cave bear remains from different profiles gives ages from 32,100 ± 1300 to >49,000 uncalibrated BP (Bieroński et al., 2009). Other extinct mammals represented in the material include the cave hyena *Crocuta crocuta spelaea*, the cave lion *Panthera spelaea*, and the bison *Bison priscus*. Paleoecological studies have indicated the co-occurrence of various ecological groups with domination of euryoecious and forest species with a much smaller contribution of steppe–tundra taxa.

In this study, we selected 39 samples of cave bear bones and teeth for DNA analyses (Table 1). They were excavated from different profiles and different parts of the cave, as shown in Fig. 2.

2.2. DNA isolation and sequencing

DNA isolation and amplification were performed as described in Baca et al. (2012). A fragment of the mtDNA control region (254 bp) was amplified with three overlapping primer pairs. Amplification products were then mixed in equimolar ratios, and sequencing

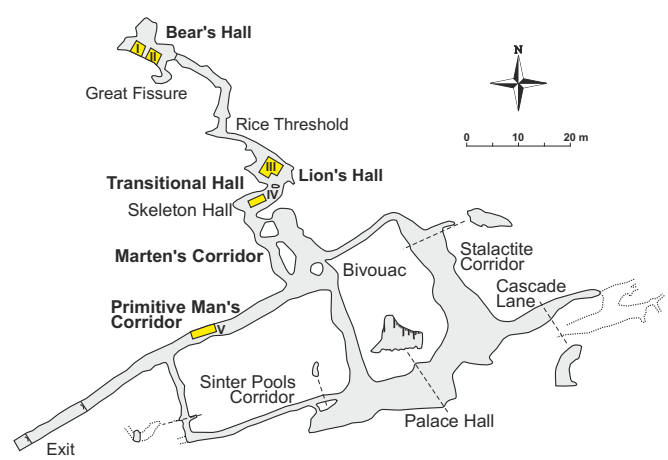

Fig. 2. Map of the central part of the Niedźwiedzia Cave. Names of the cave parts where samples were collected are bolded. Localization of excavation profiles (I–V) is indicated.

Table 1
Characteristics of cave bear samples that yielded DNA sequences.

Sample	Description	Profile[b]	Excavated in	Dating	Reference
KLE-II-16	V metacarpal	BH	Mid 70's	–	This study
KLE-III-19	II metatarsal	LH	Mid 70's	–	This study
KLE-VI-22	III metatarsal	PMC	Mid 70's	–	This study
KLE-III-24	Femur	LH	Mid 70's	–	This study
KLE-25[a]	Humerus	LH	Mid 70's	C/N ratio too high, poor collagen preservation	This study
KLE-VII-26	Tibia	LH	Mid 70's	C/N ratio too high, poor collagen preservation	This study
KK6	Long bone	MC	2010	Determination of U and Th levels failed	This study
KLE-VI-23[a]	III Metatarsal	?	Mid 70's	–	This study
KLE-IV-21	IV Metatarsal	TH	Mid 70's	–	This study
KLE-I-1	Tooth M2	BH	Mid 70's	–	This study
CB4 (KIII/220)	Tooth C	LH	Mid 70's	41,500 ± 1100 yr BP (AMS)	Baca et al., 2012
CB5 (KV)	Left ulna	PMC	Mid 70's	>49,000 yr BP (AMS)	Baca et al., 2012
10.6	Long bone	PMC	2010	87,000 ± 6000 yr BP (U–Th);>50,000 yr BP (AMS)	This study
10.4	Long bone	PMC	2010	40,000 ± 4000 yr BP (U–Th); 45,000 ± 2000 yr BP (AMS)	This study

[a] Only 240-bp sequences were obtained for these samples; thus they were not used in subsequent analyses.
[b] BH – Bear's Hall, LH – Lion's Hall, PMC – Primitive Men's Corridor, MC – Marten's Corridor, TH – Transitional Hall.

libraries were prepared according to the protocol proposed by Stiller et al. (2009), which allowed simultaneous pyrosequencing of multiple samples. The amounts of DNA molecules in each library were estimated with real-time PCR using a 454 FLX Titanium Library Quantification kit (KAPA), mixed, and sequenced on 1/8 plate region on GS FLX Titanium platform. Sequencing barcodes and primers were trimmed in BioEdit (Hall, 1999) and assembled in SeqMan Pro (DNASTAR). Consensus sequences from two replicates were called according to guidelines proposed by Stiller et al. (2009).

2.3. Phylogenetic analyses

To determine the phylogenetic position of the newly obtained sequences of cave bears from Niedźwiedzia Cave, we compared them with a set of 79 previously published cave bear sequences (Loreille et al., 2001; Hofreiter et al., 2002; Orlando et al., 2002; Hofreiter et al., 2004b; Hofreiter et al., 2007; Richards et al., 2008; Knapp et al., 2009; Stiller et al., 2010). Sequences from brown bear (*Ursus arctos*) were used as an outgroup. Phylogenies were inferred with six approaches: three Bayesian, maximum likelihood (ML), maximum parsimony (MP), and neighbor joining (NJ). Two Bayesian trees were obtained with PhyloBayes 3.2f (Lartillot and Philippe, 2004) using Poisson and CAT-Poisson models with the number of components, weights, and profiles inferred from the data. Two independent Markov chains were run for 1,000,000 generations in each of these analyses. The last 500,000 trees from each chain were collected to compute posterior consensus trees after reaching convergence. The third Bayesian tree was calculated in MrBayes (Ronquist and Huelsenbeck, 2003) using two independent runs with four Markov chains each. Trees were sampled every 100 generations for 5,000,000 generations. The final tree was calculated based on trees selected from the last 1,500,000 generations that reached the stationary phase and convergence. The ML tree was inferred in PhyML assuming two heuristic search algorithms (NNI and SPR) (Guindon and Gascuel, 2003). PAUP software (Swofford, 1998) was used to obtain MP and NJ trees. In MP analysis, the tree search was started from 10 initial trees obtained by stepwise and random sequence addition. Next, the tree-bisection-reconnection branch-swapping algorithm was applied to find the final tree.

In the MrBayes, ML, and NJ tree search, the HKY model with the portion of invariant sites was applied as indicated by jModelTest 0.1.1 (Posada, 2008). Non-parametric bootstrap analysis was performed with 1000 replications in the ML, MP, and NJ analyses. In addition to that, edge support was assessed by the approximate likelihood ratio test based on the χ^2 and the Shimodaira-Hasegawa–like procedures in PhyML.

The median-joining network (Bandelt et al., 1999), based on the 254-bp alignment of all 89 cave bear sequences, was constructed using the Network 4.6.1 software (fluxus-engineering.com). The MP algorithm was used to resolve reticulations in the final network (Polzin and Daneschmand, 2003).

2.4. Uranium–thorium dating of bone collagen

Uranium–thorium dating of bone collagen was performed in the Institute of Geological Sciences of the Polish Academy of Sciences. Bone collagen was extracted according to standard procedures described by Brown et al. (1988) and modified by Richards and Hedges (1999). Bone fragments were ground down to a fine powder in an agate mill. Sample treatment also included demineralization with 1 M HCl, gelatinization in a weak acid solution, and an ultra-filtration step. Separation of uranium and thorium isotopes was performed with the conventional chromatographic procedure with the solid ion exchanger DOWEX 1 × 8 (Ivanovich and Harmon, 1992). Activities of U and Th were determined by low-background alpha-spectrometry (Octete spectrometer by Ortec).

3. Results

3.1. Phylogenetic and sequence analyses

DNA preservation in samples from Niedźwiedzia Cave turned out to be moderate; of 39 samples screened, only 12 yielded amplifiable DNA whereas full-length sequences were obtained from 10 (Table 1). Sequences were deposited in GenBank under accession nos. KF182305 to KF182317. Together with two previously published sequences (Baca et al., 2012), seven haplotypes were identified in the analyzed material. Phylogenetic analyses revealed tree topology similar to those obtained in other studies (Knapp et al., 2009; Stiller et al., 2009; Baca et al., 2012) (Fig. 3). Three main clades can be recognized in the obtained phylogenies. One significantly supported clade consists of sequences of brown bear (*U. arctos*). Another includes sequences assigned to *Ursus deningeri kudarensis* from the Caucasus and Siberia (Yana River) (Knapp et al., 2009). This clade is rather weakly supported by most methods, and only one Bayesian approach gave very high posterior probability. However, three Caucasian sequences form a very significant cluster. The third clade contains sequences attributed to *Ursus spelaeus* and *Ursus ingressus* and is very well supported by the majority of approaches. Sequences from Conturines and Merveilleuse assigned to *U. s. ladinicus* take a basal but unsupported position. However, other sequences are grouped into two distinct

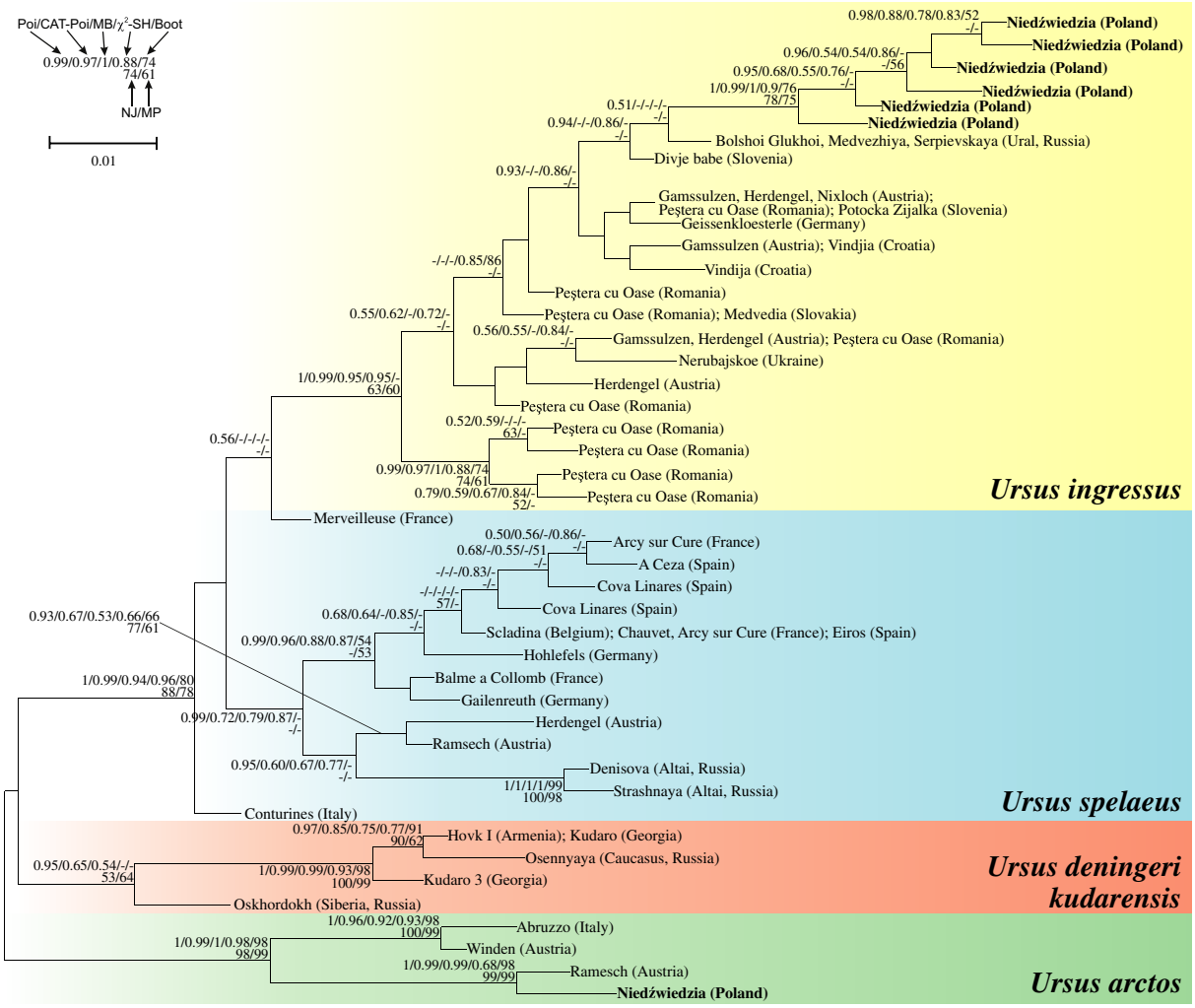

Fig. 3. Cave bear phylogenetic tree, based on mtDNA control region sequences, obtained in PhyloBayes under the Poisson model. Sequences obtained in this study are shown in bold. Values at nodes (from left to right) correspond to posterior probabilities estimated in PhyloBayes under the Poisson model (Poi), CAT-Poisson model (CAT-Poi), and MrBayes (MB) and to support values calculated in PhyML (χ^2-SH and Boot). Bootstrap values obtained in PAUP using neighbor joining (NJ) and maximum parsimony (MP) methods are shown below. Values for probabilities and bootstrap percentages lower than 0.50 and 50%, respectively, were omitted.

moderately and strongly supported clades including *U. spelaeus* and *U. ingressus* sequences, respectively.

Interestingly, one sequence obtained from a III metatarsal (KLE-VI_22) excavated in Niedźwiedzia Cave clusters with sequences of brown bear (*U. arctos*) (Fig. 3). This positioning confirms previous biometrical and morphological analyses indicating that this bear species also inhabited this cave in the Late Pleistocene (Wiszniowska, 1989; Wiszniowska et al., 1996; Bieroński et al., 2009). Eleven other sequences from Niedźwiedzia Cave cluster together with high bootstrap and posterior probability values and undoubtedly belong to the *U. ingressus* clade. They can be classified into six distinct haplotypes. These sequences are most closely related to cave bear haplotypes from the Urals (Bolshoi Glukhoi, Medvezihaya, Serpievskaya) and next to the Slovenia (Divje babe) haplotype. The support for this grouping is generally weak although one approach performed in PhyloBayes gave a 0.94 posterior probability. The branch leading to Niedźwiedzia Cave haplotypes is relatively long, which suggests a high divergence of these sequences.

The haplotype network based on all 89 cave bear sequences (Fig. 4) revealed a pattern similar to that observed on the phylogenetic tree. Four main groups corresponding to *U. arctos*, *U. deningeri kudarensis*, *U. spelaeus*, and *U. ingressus* also can be recognized. One specimen from Niedźwiedzia Cave is closely related to the brown bear whereas six other haplotypes are located in the *U. ingressus* group and connected with haplotypes from Slovenia and the Urals. None of these haplotypes was found in any other location. All of them are grouped together and are clearly separated from other *U. ingressus* haplotypes by two hypothetical ancestral haplotypes. There are five mutation steps between these haplotypes and those from Slovenia and the Urals, which form a loop (cycle) resulting probably from homoplasies and conflicting phylogenetic signal. The mean number of pairwise differences between Niedźwiedzia Cave haplotypes and other *U. ingressus* was 7.776 whereas within Niedźwiedzia Cave and the *U. ingressus* groups, the means were 2.218 and 3.016, respectively.

3.2. Dating of specimens from Niedźwiedzia Cave

In addition to the two dates obtained previously (Baca et al., 2012), we have dated four samples that yielded DNA sequences (Table 1). In the case of two samples (Kle-VII-26, Kle-25), initial C/N ratio measurements indicated that collagen preservation was poor. Two samples (10.4, 10.6) yielded both uranium–thorium and

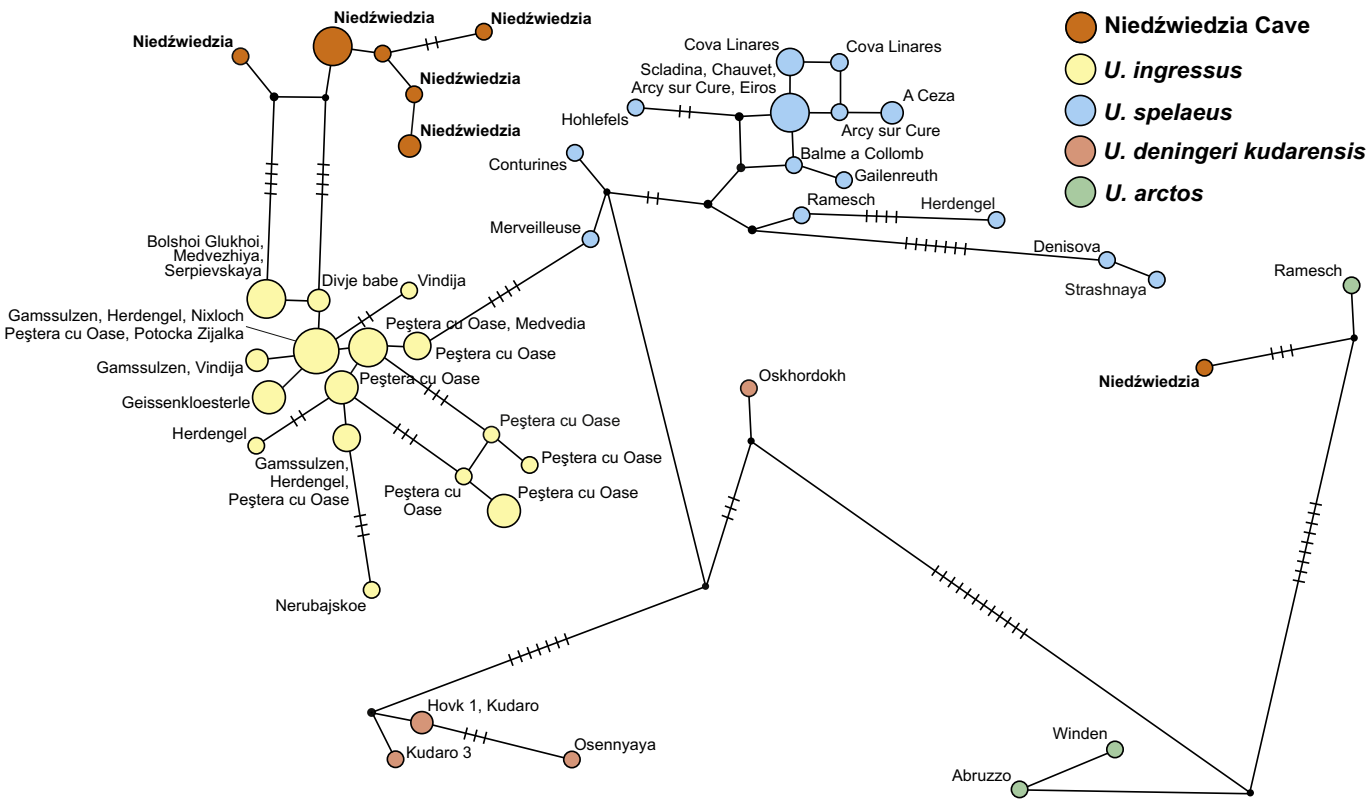

Fig. 4. Median-joining network based on 89 cave bear mtDNA control region sequences. Haplotypes are colored according to their species affiliation. Inferred missing haplotypes are presented as black dots. The area of circles is proportional to the haplotype frequency whereas the length of connecting lines corresponds to the number of substitutions between the haplotypes. If there is more than one mutation step between haplotypes, the number of mutations is presented as vertical lines.

radiocarbon dates. Sample 10.4 was dated to 40,000 ± 4000 years (U–Th) and gave a corresponding radiocarbon date of 45,000 ± 2000 BP. Sample 10.6 yielded a U–Th date of 87,000 ± 6000 years and an infinite ^{14}C date (>50,000 BP). Moreover, it was possible to estimate *terminus ante quem* for sample KK6 collected from the Martens corridor profile (Fig. 2). Calcite flowstone, which covers the entire profile, was U–Th dated to 70,000 (+20,000; −15,000) years.

4. Discussion

Paleogenetic characterization of the cave bear in Europe is still incomplete. While the distribution and phylogeography of cave bear haplotypes in Western Europe are well documented, the north and northeastern parts of its range are poorly characterized. Our analyses of aDNA from Niedźwiedzia Cave, which is located north from the Carpathian arc, at least partially fill this gap. Phylogenetic analysis of 12 DNA samples obtained from remains excavated from distinct excavation profiles of different geological ages indicated that they undoubtedly belong to the *U. ingressus* group; an exception was one sample that was clearly affiliated with the brown bear. No sequences related to *U. spelaeus* were identified, suggesting that *U. ingressus* was the only form of cave bear inhabiting the cave for at least 40,000 years. The population of *U. ingressus* from Niedźwiedzia Cave appears quite divergent from other European samples. These findings plus the age of samples (up to ca. 80,000 BP) indicate an early separation of this population from other European *U. ingressus.* Afterwards, the range of the Carpathians may have played the role of an insulating barrier because no evidence of gene flow has been identified between populations from both sides of this mountain range.

The presence of only the *U. ingressus* haplotype and the lack of *U. spelaeus* during the Early Weichselian in the Sudetes differ from other central European sites located in the Austrian Alps and Swabian Jura, to which migration of *U. ingressus* from Southeastern Europe was associated with replacement of *U. spelaeus* (Rabeder and Hofreiter, 2004; Rabeder et al., 2008; Münzel et al., 2011). It was proposed that *U. ingressus* was ecologically better adapted to continental environments and could outperform *U. spelaeus* (Baryshnikov and Puzachenko, 2011). Morphological studies of cave bears from Alpine caves revealed improved masticatory performance of *U. ingressus* in comparison to *U. spelaeus*, which allowed for more efficient food intake and processing (Rabeder et al., 2008). Greater morphological variability of *U. ingressus* also suggests its better adaptational abilities (Rabeder et al., 2008). The earliest known samples of Alpine *U. ingressus* came from Gamssulzen Cave and are dated to 47,300 BP (Bocherens et al., 2011), i.e., from the beginning of high climate fluctuations during MIS 3. This context may suggest that because of better adaptation skills, *U. ingressus* could successfully compete with *U. spelaeus* during sudden and severe climate oscillations. It cannot be excluded that these adaptations also facilitated its migration north to the Carpathians, where *U. spelaeus* was not reported. According to dating of specimens from Niedźwiedzia Cave, *U. ingressus* appeared in the Sudetes at least 20,000 years earlier than in the Austrian Alps, during MIS 5a.

In this context, it is interesting to consider if potential differences in dietary habits between *U. ingressus* and *U. spelaeus* contributed to their different distribution. It has been assumed that the cave bear was generally adapted to a herbivorous diet. The herbivory of the cave bear is well supported by many craniodental features (see e.g., Kurtén, 1976; Mattson, 1998; Rabeder et al., 2000; Grandal-D'Anglade and López González, 2004), the relatively thick

dental enamel cap (Mackiewicz et al., 2010), several adaptations in enamel structure (Wiszniowska et al., 2010), and numerous stable isotope studies (e.g., Bocherens, 1990; Bocherens et al., 1994, 2006; Nelson et al., 1998; Vila-Taboada et al., 1999; Fernández-Mosquera et al., 2001).

Isotope analyses of samples with confirmed haplotype affiliation based on DNA showed that both *U. spelaeus* and *U. ingressus* were vegetarians (Bocherens et al., 2011; Bon et al., 2011; Münzel et al., 2011). Interestingly, studies of cave bears from two closely located caves in Austria showed that *U. spelaeus eremus* and *U. ingressus* probably consumed different plant types from different habitats (Bocherens et al., 2011). Recent analyses based on large isotopic data sets revealed that the cave bear isotopic profile does not differ from that of the grizzly bear (*U. arctos horribilis*), accustomed to a diversified diet that varies from vegetarian to carnivorous (Robu et al., 2013). Moreover, samples from Southeastern European (Carpathian) sites, probably related to *U. ingressus*, showed higher values of the nitrogen stable isotope, which suggests a greater abundance of animal protein in their diet. In agreement with that, studies of *U. ingressus* samples from Greece indicated that this bear was mainly vegetarian but with a variable component of what was likely aquatic animal protein (Dotsika et al., 2011). Therefore, it cannot be excluded that *U. ingressus* was more flexible than *U. spelaeus* in its dietary habits, which facilitated its migration, especially to colder ecozones, e.g., northwards from the Sudetes and the Carpathians, as our studies have indicated.

5. Conclusions

Phylogenetic analyses of specimens from Niedźwiedzia Cave in the Sudetes showed that this cave, located north of the Carpathian arc, was inhabited for at least 40,000 years by only one taxon of cave bear, *U. ingressus*. Dating of some samples (ca. 80,000 BP) indicates that the appearance of *U. ingressus* in the Sudetes took place earlier than in Western Europe. In agreement, the haplotypes from Niedźwiedzia are divergent from those from other Western European sites. The Niedźwiedzia Cave population was probably isolated from other Western European cave bear populations because the identified haplotypes were not detected in other excavation sites.

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