

Late Pleistocene and Holocene pikas (Mammalia, Lagomorpha) from Europe and the validity of *Ochotona spelaea*: New insights based on mtDNA analysis

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ABSTRACT

Pikas were among small mammals that inhabited mammoth steppes during the last glacial. The evolutionary history of ochotonids in Europe is relatively well studied, although the taxonomic status of many described forms remains ambiguous, and the majority of extant species of the genus *Ochotona* are poorly represented in the fossil record. The present study aims to analyse the taxonomic relationships of a sample of Late Pleistocene–Holocene pikas based on mtDNA data and to clarify the status of the species described from Europe. A phylogenetic analysis has revealed that pikas form two large clades: one includes *O. pusilla* and the other includes the extant Asian and North American species. The study of haplotypes has shown similar results. The analysis supports the view that in the Late Pleistocene and Holocene *O. pusilla* was distributed throughout Europe, and its geographic range has contracted to the east until reaching its modern limits. The analysis of samples provided evidence that *O. pusilla* had survived in Eastern Europe until relatively recently and disappeared only about 150 years ago. The molecular data inferred from mtDNA do not support the species status of *O. spelaea*, despite morphological differences possibly related to the particular ecology of the Late Pleistocene.

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INTRODUCTION

During the last glacial, the so-called tundra steppe or mammoth steppe was the most extensive biome on Earth (Guthrie, 2001). The animal biomass and plant productivity of this hyperzone were similar to those of the modern African savannah (Zimov et al., 2012). The vegetation was mainly represented by grasses, herbs, and willow shrubs (Guthrie, 1990; Sher et al., 2005; Zimov et al., 2012), while deer, bison, horses, and mammoths dominated among animals (Guthrie, 2001; Kahlke, 2014). The fauna of the tundra steppe also included mammal species such as woolly rhinoceros, muskox, saiga antelope, arctic fox, wolf, dhole, cave lion, cave bear, cave hyena, various rodents (e.g., lemmings, voles, and marmots), and lagomorphs (Vereshchagin and Baryshnikov, 1992). This ecosystem covered a wide area of the Northern hemisphere, but by around 12000 years ago it had become contracted to small regions.

The mammal fauna of the tundra steppe had a non-analogue taxonomic composition (species from non-adjacent geographic zones), whose populations often showed particular morphological adaptations. The latter have at time been classified as extinct taxa that differ morphologically from their extant relatives (Rekovets, 1985, 1994, 1995). The species composing the tundra steppe fauna had a high level of specialization and unique biocoenotic relationships within the ecological communities (Rekovets and Nadachowski, 2007).

Morphological studies of fossil remains and, especially, analyses of ancient DNA allow clarification of the relationships between extinct and extant mammalian species. Such studies also help track their migration routes in Europe and Asia during the Pleistocene and Holocene (e.g., Campos et al., 2010; Baca et al., 2012; Lagerholm et al., 2014;

Doan et al., 2018). Data from palaeogenetic and phylogeographic studies allow for a deeper and more accurate taxonomic identification of Pleistocene mammals, which have so far been analysed using only morphological approaches (Niedziałkowska, 2017; Rekovets and Kovalchuk, 2017; Baca et al., 2019, 2020; Kovalchuk et al., 2021; Stefaniak et al., 2021, 2022; Doan et al., 2022; Marciszak et al., 2022; Plis et al., 2022; Ratajczak-Skrzatek et al., 2022 and many others).

The evolutionary history of the family Ochotonidae in Central and Eastern Europe is well studied (Argyropulo and Pidoplichka, 1939; Gureev, 1964; Topachevsky and Skorik, 1977; Lungu, 1981; Agadjanian and Erbajeva, 1983; Rekovets, 1985; Čermák, 2004, 2007, 2010; Čermák and Rekovets, 2010; Fostowicz-Freluk and Freluk, 2010; Čermák, 2016). The extinct taxa that occurred in these European regions from the Miocene to the Pleistocene have been identified as separate species within the genera *Prolagus*, *Pliolagomys*, *Ochotonoma*, and *Ochotona*. The taxonomic status of many of these forms is however still debated despite the fact that the morphology of their remains has been studied in detail. The genus *Ochotona* has been represented in the Palaeoarctic since the early Late Miocene (Čermák, 2016).

Ochotonids appeared in Asia between the Late Eocene and Early Oligocene and continued to evolve along with the distribution of C₃ grasses in previously forest-dominated areas through the 'climatic optimum' from the Late Oligocene to Middle Miocene (Ge et al., 2013). They thrived in Eurasia, North America, and Africa. The peak of their diversity occurred during the period from the Early to Middle Miocene. Most pikas became extinct during the transition from the Miocene to Pliocene, which was accompanied by an increase in the diversity of

leporids (Erbajeva et al., 2011). This switch between ochotonids and larger leporids was probably caused by the expansion of C₄ plants due to global cooling in the Late Miocene, since extant pikas show a strong preference towards C₃ plants (Ge et al., 2013). *Ochotona* is the sole extant genus within the family, although many fossil genera have been described (Erbajeva et al., 2011). The genus *Ochotona* includes 34 living species, most of which are restricted to China. Only one species — the steppe pika, *Ochotona pusilla* Pallas, 1768 — is today represented in the fauna of Europe (in particular, in southern Russia and northern Kazakhstan), while the American pika *Ochotona princeps* (Richardson, 1829) and collared pika *Ochotona collaris* (Nelson, 1893) occur in North America (Hoffmann and Smith, 2005).

The genus *Ochotona* includes a number of extinct species described from Pliocene and Pleistocene deposits of Europe and Asia. At the same time, the majority of extant species of this genus (including *O. pusilla*) are relatively poorly documented in the fossil record, especially for the last several thousand years (Fostowicz-Frelik and Frelik, 2010).

The systematics of the genus *Ochotona* based on both morphological and molecular data is therefore unstable and is subject to changes (Niu et al., 2004; Čermák, 2010; Lissovsky et al., 2013). An especially noteworthy Late Pleistocene species *Ochotona spelaea* was erected by Richard Owen, the famous nineteenth century British anatomist. *O. spelaea*, originally *Lagomys spelaeus*, was named on the basis of material from the Late Pleistocene deposits of Kent's Cavern, England, and described as having different cranial and dental characters.

Systematic relationships within the genus *Ochotona* have been recently updated due to the inclusion of a large number of extant species into morphological analyses (Hoffmann and Smith, 2005; Erbajeva et al., 2011; Reese et al., 2013; Lissovsky, 2014) as well as the use of molecular data and up-to-date statistical methods (Yu et al., 1997, 2000; Niu et al., 2004; Lissovsky et al., 2007; Lanier and Olson, 2009). At the same time, DNA sequences of the genus *Ochotona* derived from fossil remains of its representatives remain to be studied.

The present study sheds light on the phylogeny of Late Pleistocene and Holocene pikas based on mtDNA data. We also assess the likely taxonomic status of the extinct European species *Ochotona spelaea* (Owen, 1846) that has been

inconsistently identified from Late Pleistocene localities in Europe. The type material of *O. spelaea* from Kent's Cavern is not available to sample and validate the species so instead we investigate coeval material from the British Isles and Belgium in addition to a single specimen from Novgorod-Siverskyi in Ukraine that was identified as *O. spelaea* by Rekovets (1985). The material from Britain and Belgium has been identified merely as *Ochotona* sp. in recognition of the uncertainties over the Late Pleistocene pika identifications in Europe and because it was not analysed morphologically.

MATERIAL AND METHODS

Sampling and Sample Preparation

Cytochrome b mitochondrial DNA sequences were obtained for 24 individuals of the genus *Ochotona* (Table 1), ranging in length from 250 to 510 bp. These specimens were previously assigned to *O. pusilla* (N=9) or *O. spelaea* (N=1) based on morphological characters, and *Ochotona* sp. (N=14) for the material that was not analysed morphologically in detail. Sequences of various *Ochotona* species available in the GenBank database (39 specimens, 14 species) were also included in the analysis (Table 2). The sequences of *Microtus pennsylvanicus* and *Peromyscus boylii* were used for rooting the phylogenetic tree. The comparative sequences taken from the GenBank database belong to recent individuals. Eighteen of the 24 individuals whose sequences were produced in this study date back to 550–20,000 years while the other six individuals from Kazakhstan, Russia and Kozatske in Ukraine are much younger (Table 1).

The mtDNA of all specimens of the genus *Ochotona* was obtained using the method described in Ersmark et al. (2015). After cleaning the fossil remains, the outer surface of each bone has been removed and a sample (10–50 mg of bone powder) was obtained with a tungsten drill. Bone powder was then placed in a 1.5 ml Eppendorf tube, to which the following reagents were successively added: 630 µl EDTA (0.5 M, pH 8), 70 µl UREA, and 15 µl proteinase K (10 µg/µl). The samples were incubated for about 24 hours in a mobile incubator at 55°C and then centrifuged at 2300 rpm for 5 min. The supernatant was then transferred to Vivaspin® columns and centrifuged at 12,000 rpm for 7–10 min (until 120–100 µl or less of fluid has left in the filter). The obtained sample mix was transferred using fine tips to a 1.5 ml tube containing 600 µl of PB buffer. The entire

TABLE 1. Details of the *Ochotona* samples analysed in this study. Asterisks indicate the specimens donated by A. Liss-ovsky (*) and N. Abramson (**). The absolute age of the specimens in years: 20,000 (1), 150 (2), 550 (3), 36 (4), 17,000 (5), 9915 ± 60 BP (OxA8073) after Ramsey et al. (2002) and Price (2003) (6).

| No. | Taxon (initial ID) | Country | Locality | Age | Reference |
|-----|-------------------------|------------|---------------------------------|---------------|-------------------|
| 1* | <i>Ochotona pusilla</i> | Kazakhstan | – | Recent | This paper |
| 2* | <i>Ochotona pusilla</i> | Russia | – | Recent | This paper |
| 3** | <i>Ochotona pusilla</i> | Russia | – | Recent | This paper |
| 4** | <i>Ochotona pusilla</i> | Russia | – | Recent | This paper |
| 5 | <i>Ochotona spelaea</i> | Ukraine | Novgorod-Siverskyi ¹ | Last Glacial | Rekovets, 1985 |
| 6 | <i>Ochotona pusilla</i> | Ukraine | Kozatske ² | Late Holocene | Pidoplichka, 1934 |
| 7 | <i>Ochotona pusilla</i> | Ukraine | Raiky ³ | Late Holocene | Pidoplichka, 1934 |
| 8 | <i>Ochotona pusilla</i> | Russia | Orenburg ⁴ | Recent | This paper |
| 9 | <i>Ochotona pusilla</i> | Ukraine | Mezhyrich ⁵ | Last Glacial | Rekovets, 1985 |
| 10 | <i>Ochotona pusilla</i> | Ukraine | Mezhyrich ⁵ | Last Glacial | Rekovets, 1985 |
| 11 | <i>Ochotona</i> sp. | Belgium | Rocher de Nevioux | Last Glacial | This paper |
| 12 | <i>Ochotona</i> sp. | Belgium | Rocher de Nevioux | Last Glacial | This paper |
| 13 | <i>Ochotona</i> sp. | Belgium | Rocher de Nevioux | Last Glacial | This paper |
| 14 | <i>Ochotona</i> sp. | Belgium | Rocher de Nevioux | Last Glacial | This paper |
| 15 | <i>Ochotona</i> sp. | Belgium | Rocher de Nevioux | Last Glacial | This paper |
| 16 | <i>Ochotona</i> sp. | England | Bridget Pot | Younger Dryas | This paper |
| 17 | <i>Ochotona</i> sp. | England | Bridget Pot | Younger Dryas | This paper |
| 18 | <i>Ochotona</i> sp. | England | Bridget Pot | Younger Dryas | This paper |
| 19 | <i>Ochotona</i> sp. | England | Bridget Pot | Younger Dryas | This paper |
| 20 | <i>Ochotona</i> sp. | England | Bridget Pot | Younger Dryas | This paper |
| 21 | <i>Ochotona</i> sp. | England | Merlin's Cave ⁶ | Younger Dryas | This paper |
| 22 | <i>Ochotona</i> sp. | England | Merlin's Cave ⁶ | Younger Dryas | This paper |
| 23 | <i>Ochotona</i> sp. | England | Merlin's Cave ⁶ | Younger Dryas | This paper |
| 24 | <i>Ochotona</i> sp. | England | Merlin's Cave ⁶ | Younger Dryas | This paper |

sample was vortexed and centrifuged. The sample/PB mix was then placed into a QIAquick column (QIAGEN) and centrifuged at 13,000 rpm for 1 min. After the residual buffer was removed, 720 µl PE buffer was added to the column and centrifuged at 13 000 rpm for 1 min, then the PB buffer was removed and centrifuged again. The column was transferred to 1.5 ml Eppendorf tubes and allowed to dry for about 5 min with the lids open. Then 50 µl of EB buffer was added directly to the filter. It was left for 1 min and centrifuged at 13 000 rpm for 1 min. Another 50 µl of EB buffer was added directly to the filter. It was left for 1 min and centrifuged at 13 000 rpm for 1 min. The DNA extract was transferred to a new 1.5 ml tube with a screw cap. The extract obtained in this way was amplified within cytochrome b with seven short overlapping fragments, approximately 500 bp long. After amplification, the DNA was purified and checked on agarose gel to verify the PCR results. The posi-

tively verified samples were sequenced in both directions on an ABI 3730XL sequencer. The study of the samples (determination, purification, milling, isolation, amplification, sequencing, and bioinformatic analysis) was carried out in the Department of Bioinformatics and Genetics, Swedish Museum of Natural History in Stockholm.

Amplification of the Cytochrome b Fragment

Ancient DNA is highly fragmented thus it is recommended to use primer pairs for a few relatively short fragments (Poinar, 2003), product length ca. 80–200 bp (Table 3). Additionally, in order to verify the results, at least three replications of the PCR and sequencing reactions were performed for each reaction. The PCR reaction was prepared in a volume of 25 µl. It was performed with 2.5 µl of PCR buffer, 0.5 µl of each primer (10 µM), 0.5 µl of dNTP (10 µM), 1 µl of MgCl (2.5 mM), 2.5 µl of BSA (1 mg/ml), 0.4 µl of Hot Star

TABLE 2. The list of specimens from GenBank used to compare the obtained results.

| Species | GenBank | Species | GenBank |
|----------------------------|----------|------------------------------------|----------|
| <i>Ochotona alpina</i> | AF273009 | <i>Ochotona princeps</i> | EU590921 |
| <i>Ochotona alpina</i> | DQ335517 | <i>Ochotona princeps</i> | EU590922 |
| <i>Ochotona alpina</i> | EF567055 | <i>Ochotona pusilla</i> | AY260744 |
| <i>Ochotona alpina</i> | HM346527 | <i>Ochotona pusilla</i> | HM366945 |
| <i>Ochotona alpina</i> | HM346529 | <i>Ochotona pusilla</i> | JX682567 |
| <i>Ochotona collaris</i> | EU549738 | <i>Ochotona pusilla</i> | JX682568 |
| <i>Ochotona collaris</i> | JQ624503 | <i>Ochotona roylei</i> | JX682573 |
| <i>Ochotona collaris</i> | JQ624508 | <i>Ochotona rutila</i> | JX682566 |
| <i>Ochotona collaris</i> | KP411020 | <i>Ochotona thibetana</i> | AF272986 |
| <i>Ochotona erythrotis</i> | AF272999 | <i>Ochotona thibetana</i> | KU359597 |
| <i>Ochotona forresti</i> | AF272998 | <i>Ochotona thibetana</i> | KU359598 |
| <i>Ochotona huangensis</i> | JN645147 | <i>Ochotona thibetana</i> | KU359599 |
| <i>Ochotona hyperborea</i> | AB053257 | <i>Ochotona thibetana</i> | KU359600 |
| <i>Ochotona hyperborea</i> | KR076818 | <i>Ochotona thomasi</i> | AF272987 |
| <i>Ochotona hyperborea</i> | KR076820 | <i>Ochotona thomasi</i> | KU359612 |
| <i>Ochotona hyperborea</i> | KR076821 | <i>Ochotona thomasi</i> | KU359613 |
| <i>Ochotona hyperborea</i> | KR076823 | <i>Ochotona thomasi</i> | KU359614 |
| <i>Ochotona koslowi</i> | AF272993 | <i>Ochotona thomasi</i> | KU359615 |
| <i>Ochotona macrotis</i> | AF273010 | *Sequences added to root the tree: | |
| <i>Ochotona macrotis</i> | JQ670914 | * <i>Microtus pennsylvanicus</i> | KC473477 |
| <i>Ochotona macrotis</i> | JX682574 | * <i>Peromyscus boylii</i> | AF155387 |

polymerase (Qiagen, Germany), distilled water, and the appropriate volume of DNA depending on the quality and concentration of the template. The mixtures prepared in this way were placed in the Eppendorf Mastercycler EP thermocycler and amplified using the following program: 95°C — 10 min, then 55 cycles [94°C — 30 sec., 50–52°C (depending on the primer) — 30 sec., 72°C — 30 sec.], 72°C — 7 min, 12°C 'for ever'. The next step was a comprehensive bioinformatic and phylogenetic analysis of the results of sequencing of the mitochondrial genome fragments obtained from fossils together with data on extant species.

The obtained sequences were compared with the available cytochrome b sequences of selected representatives of the genus *Ochotona* (Yu et al., 2000; Takaki et al., 2001; Niu et al., 2004; Galbreath et al., 2009; Grigorieva et al., 2010; Kartavtseva et al., 2014; Lanier et al., 2015; Lissovsky et al., 2007, 2008; Lanier and Olson, 2009, 2013; Lissovsky, 2014; Liu et al., 2017). The sequence numbers used to compare and verify the obtained results are presented in Table 2.

Three replicates of the PCR for each specimen and each primer were aligned, and a consensus sequence was constructed on this basis. Then

the sequences from both primers for the same DNA fragment were aligned, and a consensus sequence was created for a given primer pair. The final step was to align the sequences of all primers into the final consensus sequence for a given specimen. The sequences obtained in this way for all individuals, combined with homologous sequences obtained from the GenBank database, were compared using the Muscle algorithm (Edgar, 2004) implemented in the Seaview program (Gouy et al., 2010).

After alignment, the analysed sequences were truncated in order to obtain a compact block of DNA. The sets of sequences prepared in this way were used to create phylogenetic trees using the Maximum Likelihood and Bayesian Analysis methods. For analyses with the highest likelihood method, the PHYML program was used with the implemented smart model selection option for automatic selection of the substitution model (Guindon et al., 2010). The tree topology was verified with bootstrap analysis for 1000 replicates. The program MrBayes 3.2 was used to create the trees using the Bayesian method (Ronquist et al., 2012). The jModelTest 2.1.10 application was used to estimate the best-fit nucleotide substitution mod-

TABLE 3. Primer pairs used to amplify the ancient mtDNA control-region sequence of the cytochrome b.

| Primer name | Primer sequences (5'-3') | Size (bp) | Ta*, °C |
|-------------------------|--------------------------|-----------|---------|
| Och1f | AAACCACCCCCTAATAAAAA | 143 | 50 |
| Och1r | GTATGGCTAGGAATAAGCC | | |
| Och2f | CCGCATGATGAAACTTCGGA | 121 | 50 |
| Och2r | TGGGTGACTGATGAGAATGC | | |
| Och3f | TCCTAGCCATACACTACACA | 121 | 50 |
| Och3r | AATATGGATGCGCCGTT | | |
| Och4bf | TAAACTACGGCTGAATTATCC | 129 | 52 |
| Och4br | CCGATGTTTCATGTTTCTGAG | | |
| Och5f | CTATTCCTACACGTAGGCCG | 141 | 50 |
| Och5r | GAAGGATATTTGTCCTCATGG | | |
| Och6bf | TAGCAACCGCCTTCATAGG | 131 | 52 |
| Och6br | AGCCGCCTCAGATTCATTG | | |
| Och7bf | CCATGAGGACAAATATCCTTC | 127 | 52 |
| Och7br | GTGAGGGTAGCTTTATCAACTG | | |
| Recent specimen (ER008) | | | |
| Och1f | AAACCACCCCCTAATAAAAA | 143 | 50 |
| Och1r | GTATGGCTAGGAATAAGCC | | |
| Och2f | CCGCATGATGAAACTTCGGA | 121 | 50 |
| Och2r | TGGGTGACTGATGAGAATGC | | |
| Och3f | TCCTAGCCATACACTACACA | 121 | 50 |
| Och3r | AATATGGATGCGCCGTT | | |
| Och4f | ACGGCTGAATTATCCGCTAC | 115 | 50 |
| Och4r | CATGTTTCTGAGTAGGTGTA | | |
| Och5f | CTATTCCTACACGTAGGCCG | 141 | 50 |
| Och5r | GAAGGATATTTGTCCTCATGG | | |
| Och6f | CCGCCTTCATAGGCTAC | 121 | 50 |
| Och6r | CCTCAGATTCATTGGAC | | |
| Och7f | AACCGTAATTACCAACCTCC | 113 | 50 |
| Och7r | AGGCAAAGAATCGAGTTAGG | | |

* Annealing temperature

els used in the MrBayes program (Darriba et al., 2012). For protein coding sequences (Cytb), separate models were selected for each letter in the codon — *Ochotona* sp.: HKY + I + G for I, II, and III letters in the codons. During the analysis in the MrBayes program, we used the option of two independent runs each consisting of four Markov chains. The trees were sampled every hundredth generation for 20,000,000 generations (using 25% burn-in values). The analysis was completed when the standard deviation of the mean log-probability values of both robots stabilized at a level below 0.01 for all trees used to create the final tree. In many cases, standard tree-based phylogenetic analysis is unable to fully resolve close phylogenetic relationships; therefore, a haplotype network

was made for individual sequences. The Median Joining method (Bandelt et al., 1999) implemented in the PopART 1.7 program (Leigh and Bryant, 2015) was used to create the network. For their implementation, the same data sets were used as in the case of trees.

RESULTS

The obtained results combined with data from previous molecular analyses allowed the construction of a phylogenetic tree and haplotype network for pikas in order to study their systematic relationships. We suggest that the position of *Ochotona* individuals from the Pleistocene of Europe in the phylogenetic tree based on cytochrome b analysis

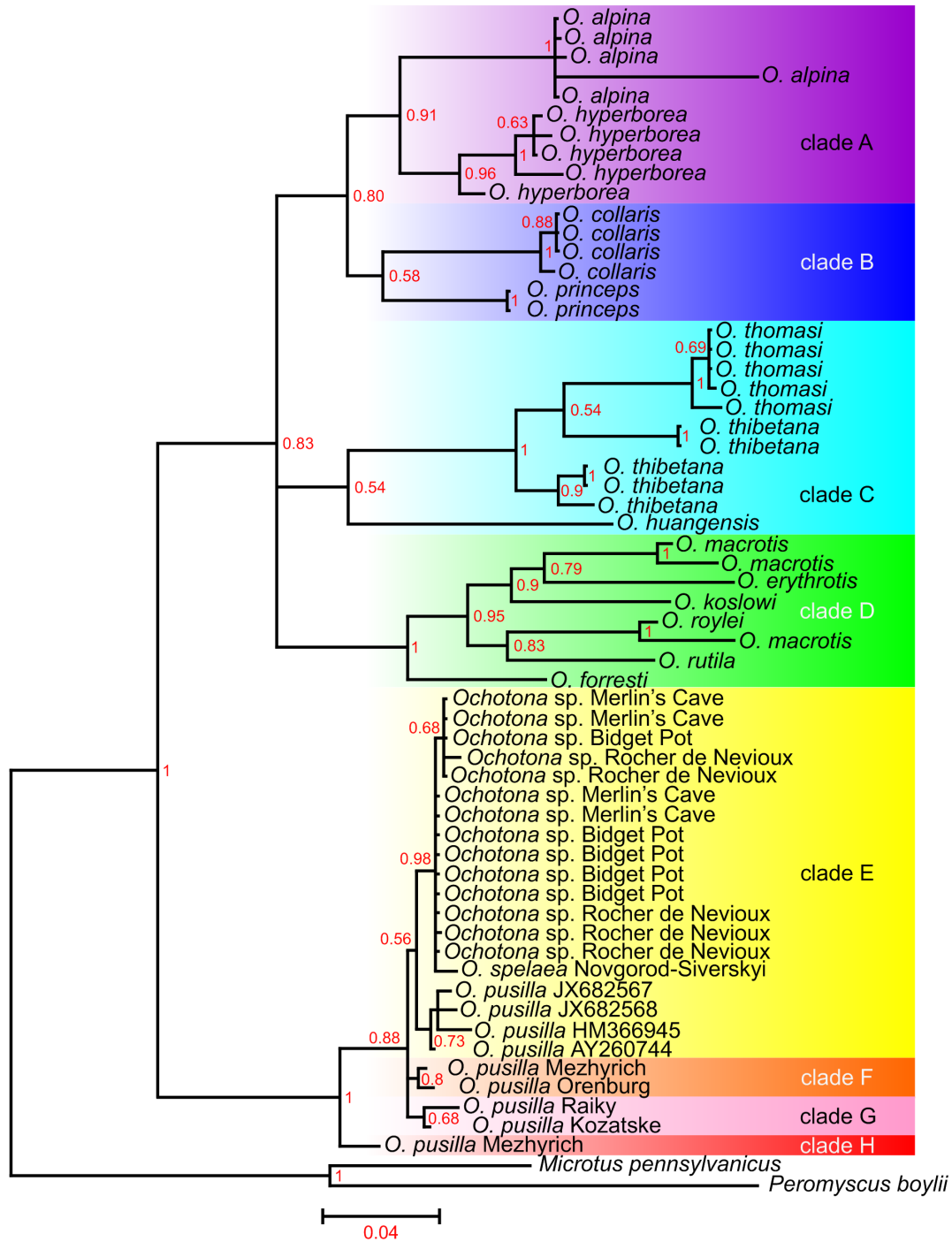


FIGURE 1. Phylogenetic tree of the genus *Ochotona* based on the analysis of cytochrome b mtDNA sequences.

is reliable, considering the significance level (> 0.80) of all nodes (Figure 1).

The phylogenetic tree of the genus *Ochotona* (Figure 1) shows two main branches. The first branch includes the clades A+B, C, and D (prob-

ability level 0.83), which represent the Asian (except for *O. pusilla*) and American species. Individuals of *O. alpina* (Pallas, 1773) and *O. hyperborea* (Pallas, 1811) form clade A, while *O. princeps* and *O. collaris* from North America form clade B. Clade C

includes individuals belonging to *O. thomasi* Argyropulo, 1948 and *O. thibetana* (Milne-Edwards, 1871) together with *O. huangensis* (Matschie, 1908), which is however separated from the two above taxa. All three species of clade C almost inhabit the same area — the central part of China. The last branch (clade D) consists of South Asian species: *O. macrotis* (Günther, 1875), *O. erythrotis* (Büchner, 1890), *O. koslowi* (Büchner, 1890), *O. roylei* Ogilby, 1839, *O. rutila* (Severtzov, 1873), and *O. forresti* Thomas, 1923.

The second main branch includes the specimens analysed in this study (i.e., *O. pusilla*, *O. spelaea*, and *Ochotona* sp.) together with individuals of *O. pusilla* taken from the GenBank (clades E, F, and G). Within this second main branch, one specimen from the Late Pleistocene of Mezhyrich, Ukraine, forms a separate clade H. Two possible explanations are that either the two fossils represent a contact zone between two clades at one time or the fossils, which have not been dated, are not precisely the same age and a population turnover took place. The clades F and G within this group are represented by specimens of Holocene age (Kozatske, Raiky, and Orenburg). The specimens of *O. pusilla* from the GenBank along with the remaining fossil specimens form clade E. Apart from the specimen of *Ochotona spelaea* from Novgorod-Siverskyi, they come from the Last Glacial of England and the Younger Dryas of Belgium. Such a close connection to *O. pusilla*, of the studied individuals from Ukraine, Russia, and Western Europe, suggests that they represent the same species — *O. pusilla* (Figure 1).

The cytochrome b sequences of the individuals analysed in this study do not differ from the respective sequence of recent *O. pusilla* from the GenBank. The cytochrome b sequences ranging in length from 250 to 510 bp and those from the GenBank were used to build the haplotype network of the genus *Ochotona* (Figure 2). The specimens of *O. pusilla*, *O. spelaea*, and *Ochotona* sp. created a system of four closely related haplotypes: Haplotype 1 (samples 6–7, 11–24 in Table 1), Haplotype 2 (sample 5 in Table 1), Haplotype 3 (samples 8 and 9 in Table 1), and Haplotype 4 (sample 10 in Table 1). They show a low number of substitutions compared to the modern *O. pusilla* haplotype, which suggests that they should be assigned to the same species (Group 5, pink). The haplotypes of *O. macrotis*, *O. koslowi*, *O. erythrotis*, and *O. roylei* together form a related group (Group 1, blue), showing little difference between particular sequences. *O. rutila* is also associated with the

mentioned group to a greater extent than the other species. It is the node connecting this group with the rest. The haplotypes of *O. thomasi* and *O. thibetana* (both occurring in the central part of China) show a small number of substitutions (Group 2, yellow). The other haplotypes of the analysed species form two interrelated groups. Group 3 (green) represents the American species *O. collaris* and *O. princeps*, related to each other. The species *O. alpina* and *O. hyperborea* form Group 4 (azure). *O. huangensis* (Southeast Asia) and *O. forresti* (Southwest Asia) have more point changes and are therefore distant from the rest of the species. However, unlike the phylogenetic tree, in the haplotype network *O. huangensis* is related to *O. princeps* and *O. hyperborea*. In the tree, this species is associated with the *O. thibetana/thomasi* group. On the other hand, *O. forresti* in the tree is associated with the *O. macrotis/erythrotis/koslowi/roylei/rutila* group, while in the haplotype network it is associated with the *O. alpina/hyperborea* group (Figure 2). Nevertheless, the results of the analysis of the phylogenetic tree and the haplotype network are generally very similar.

DISCUSSION

Pikas have high intraspecific morphological diversity and low interspecific diversity (Lanier and Olson, 2009). This makes it difficult to use morphological data to understand the evolutionary history of the different species. The rate of change in cytochrome b sequences within and between taxa (Irwin et al., 1991) allows its use in evolutionary studies of *Ochotona* (Yu et al., 2000). The topology of the phylogenetic tree, based on cytochrome b sequences, for individuals in the genus *Ochotona*, is similar to that in the most recent systematic analysis (Lissovsky, 2014). The division of the phylogenetic tree into two main branches — Asian and American species (A–D) and *Ochotona pusilla* (E–H) respectively — is in line with previous results. The position of *O. pusilla* in the phylogenetic tree in Lissovsky (2014), where it is classified as basal for the genus, is identical to that obtained in our study. The relationship between *O. alpina* and *O. hyperborea* within clade A is confirmed in the systematics of the genus *Ochotona* (Lissovsky et al., 2007; Lissovsky, 2014). These species are similar to the North American *O. collaris* and *O. princeps*, which form clade B (Lissovsky et al., 2007) previously described as the Nearctic subgroup (Yu et al., 2000; Niu et al., 2004). These relationships are consistent with the geographic range of these groups, covering the vicinity of the Bering Strait,

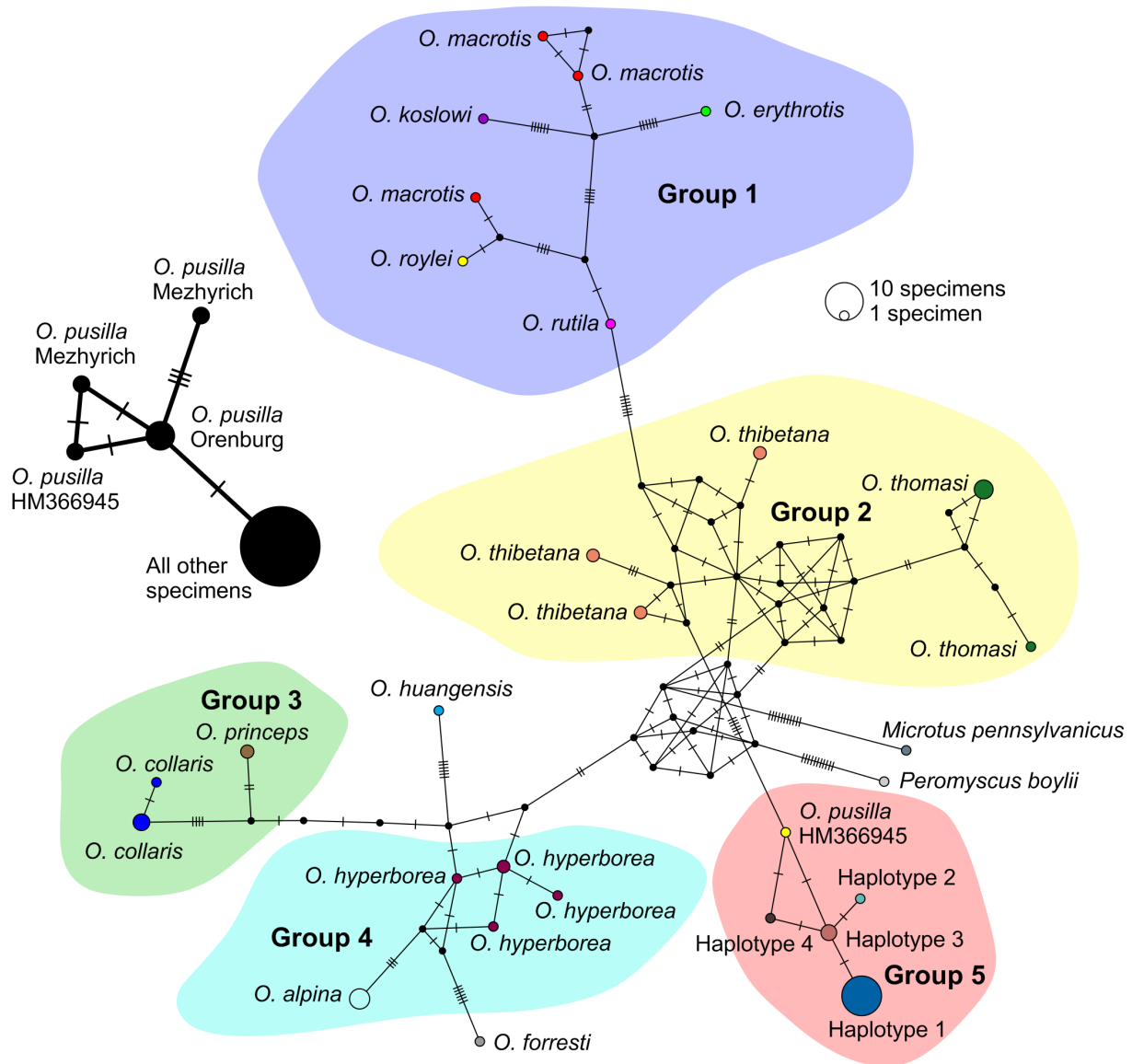


FIGURE 2. Haplotype network of species of the genus *Ochotona* based on the analysis of cytochrome b mtDNA sequences.

which was a dispersal route between North America and Asia at times of low sea level.

The first representatives of the *O. pusilla* group, which were widely distributed in Europe, appeared in the Palearctic at the Plio-Pleistocene boundary (Erbajeva et al., 2001). The steppe pika is regarded a relict species of the Late Pliocene faunas of Eurasia based on its fossil record, molar structure, karyological traits, and mtDNA sequence data (Németh et al., 2017). Representatives of this group were characterised by a number of plesiomorphic traits, in particular their smaller body size

and the large distance between the anteroconid and posteroconid of the p3 (Erbajeva et al., 2011). At present, the steppe pika is distributed in Central Asia, east of the Urals throughout southern Russia and northern Kazakhstan. During the Pleistocene, however, its range was broader and included much of Europe (Laplana et al., 2015), and later contracted to the east (Erbajeva, 2008). *Ochotona pusilla* survived in the Carpathian region until 5,000–4,000 years BP (Németh et al., 2017), in the central Urals until the Middle Holocene, and in the

southern Urals until the Late Holocene (Kosintsev and Bachura, 2014).

Pidoplichka (1934) found 550-year-old remains of *O. pusilla* in Raiky, Ukraine, as well as specimens dated to the nineteenth century in Kozatske, Ukraine. He suggested that the species had existed in Europe until that time. However, on the map showing the species' range (Pidoplichka, 1934: fig. 2), he marked individuals from Central and Western Europe as extinct *O. pusilla*. The age of the steppe pika remains from the localities of Raiky and Kozatske is estimated as Late Holocene (Pidoplichka, 1934). The suggested presence of *O. pusilla* in Ukraine can be considered correct due to the analysis of Holocene remains presented here. According to Pidoplichka (1934) and Zagorodniuk (2016), *O. pusilla* disappeared in Ukraine about 100–150 years ago, and the geographic range of the species continued to decrease eastward.

Rekovets (1985) had identified *Ochotona* remains from the Late Pleistocene of the Crimea, Kostenki-on-Don, and other Pleistocene sites as *O. spelaea*, which he considered an index species of periglacial faunas of Europe (together with *Lepus tanaiticus*, *Spermophilus superciliosus*, and *S. severskensis*). *Ochotona spelaea* was described as morphologically similar to *O. pusilla* (except for having broader frontals, more developed zygomatic arch, and simpler p3) and thought to be phylogenetically closely related (Rekovets, 1985). According to Erbajeva (1988) and Averianov (2001), small pikas from the Middle and Late Pleistocene of Europe (except for those from the Crimea) should be assigned to the subspecies *Ochotona pusilla spelaea*. This would signify that the species has both a large geographic and temporal range and includes a number of localities of Middle Pleistocene age, such as Varbezhnitsa in Bulgaria, Belvédère in the Netherlands, La Fage in France (Chaline, 1975; Kolfshoten, 1985; Popov, 1988). Those dated to the Late Pleistocene include: Niksloch in Austria, Gornja Bijambarska in Bosnia and Herzegovina, Veternica and Pecine u Brini in Croatia, Pillisszántó and Subalyuk in Hungary, Starye Duruityory and Brynzeny-1 in Moldova, Zalasie in Poland, Smolucka Cave in Serbia, Novgorod-Siverskyi and Mezhyrich in Ukraine (Kormos, 1916; Mottl, 1938; Lozan, 1970; Malez, 1963, 1966, 1968, 1979; Bocheński et al., 1985; Dimitrijević, 1988; Rekovets and Topachevsky, 1988; Fladerer, 1992). Other researchers such as Rekovets (1985) believed that *O. spelaea* was a separate species that became extinct in Europe ca. 10 000 years ago.

The mitochondrial DNA sequences of the 24 *Ochotona* samples originating from the Pleistocene and Holocene of Europe and analysed in this study based on cytochrome b showed their considerable similarity with that of *O. pusilla*. The clade formed by the analysed specimens together with representatives of *O. pusilla* is clearly distinguished from all the other species used in the comparison. We can therefore suggest that *O. pusilla* was the only pika species that inhabited Europe during the Pleistocene. As a consequence, the specimens previously assigned to *O. spelaea* most probably belong to *O. pusilla*. The different morphology of the Late Pleistocene material may be a distinct set of ecomorphological traits that have no taxonomic significance. Other vertebrates have been shown to have similar morphologically distinct populations in Late Pleistocene that have no taxonomic significance and may relate to the rich ecology of the tundra steppe (e.g., Lister et al., 1987; Stewart, 1999; Meiri et al., 2013; Lagerholm et al., 2017). The possibility that they represent a separate subspecies based on morphological data (Erbajeva, 1988; Averianov, 2001) cannot be sustained by the phylogenetic analysis as they are too closely related to modern individuals of *O. pusilla*. *Ochotona spelaea* is therefore a chronospecies that has been named for the Late Pleistocene material of Europe.

It seems therefore that during the Pleistocene, *O. pusilla* had a wide intraspecific variability and its representatives dispersed over the vast steppe areas of Europe and Asia (Erbajeva et al., 2011). Climatic changes, the development of forests in the post-glacial era, and the disappearance of grasslands during the Holocene have significantly limited the range of this species (Shubin, 1963; Erbajeva et al., 2011). Human economic activity (in particular, the plowing of virgin lands) is also considered one of the factors that led to the destruction of natural habitats of *O. pusilla* and contributed to disappearance of this species in most of Eastern Europe (Sokur, 1961; Lukyanov, 2001). The results of the mtDNA analysis have also confirmed that the steppe pika occurred in the territory of modern Ukraine about 150 years ago (Pidoplichka, 1934; Pidoplichko, 1973; Zagorodniuk, 2016), although the current range of the species is mainly restricted to the steppes of Central Asia (Erbajeva et al., 2001). It is hypothesized that the steppe pika could disappear from the territory of Ukraine due to zoonoses of pestis in combination with other factors (Zagorodniuk, 2016), although such an assumption needs to be additionally verified.

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