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Source: Journal of Vertebrate Biology, 74(24119)

Published By: Institute of Vertebrate Biology, Czech Academy of Sciences

URL: https://doi.org/10.25225/jvb.24119

Journal of Vertebrate Biology

Open Access

J. Vertebr. Biol. 2025, 74: 24119 DOI: 10.25225/jvb.24119

RESEARCH PAPER

Recolonisation or invasion? First insights into the origin of the newly established beaver populations in Bulgaria using eDNA

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Received 8 November 2024; Accepted 21 March 2025; Published online 17 April 2025

Abstract. After a series of reintroductions (including unofficial releases) and natural recolonisations during the last decades, the Eurasian beaver (*Castor fiber*) is returning throughout Europe. However, it is hard to distinguish the species from its American relative, *Castor canadensis*, which has also been successfully introduced and is thriving in some parts of the continent as an invasive species. In this context, obtaining genetic information for the newly recorded populations is important for taking appropriate management actions. The current paper presents the results of the first genetic assay of five recently established beaver populations along the River Danube and two of its tributaries in Northern Bulgaria. The non-invasive method of eDNA sampling was applied. We found that all populations belong to *C. fiber* and are of the haplotypes from the refugial populations in Norway and France. We hypothesise that they naturally spread from Romania, where reintroductions have been done using individuals from Bavaria, who were, in turn, translocated from Scandinavia, France, Russia, and Poland.

Key words: genetic monitoring, *Castor fiber*, mammal conservation, Danube

Introduction

The Eurasian beaver, *Castor fiber* L., 1758, is the largest rodent in Europe and Asia (Rosell et al. 2005). Formerly, it was abundant throughout Eurasia, but between the 16th and 19th centuries, habitat loss and over-hunting for the fur and perfume trade severely impacted its populations (Nolet & Rosell 1998) to the point of extirpation from a great part of its former range (Halley et al. 2021), including Bulgaria (Boev & Spassov 2019). Nowadays, the beaver is considered a keystone species and a riparian ecosystem engineer because of its unique behaviour, which disproportionately impacts ecosystem structure

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and function, creating habitats for various species (Janiszewski et al. 2014, Brazier et al. 2021). Beavers contribute to multiple ecosystem services such as greenhouse gas sequestration, water purification, flood mitigation, nutrient cycling, recreational hunting and fishing, water supply, and ecotourism (Brazier et al. 2021, Thompson et al. 2021). This modern recognition led to multiple successful reintroductions throughout Europe (Frosch et al. 2014, Mai et al. 2018). The subsequent natural spread assured the recovery of the species, which is now marked as 'Least Concern' in the IUCN Red List database (Batbold et al. 2021). Unfortunately, some translocations involved its North American congener *C. canadensis* Kuhl, 1820 (Dewas et al. 2012), which has successfully established populations in Finland and Karelia, Russia (Parker et al. 2012, Fyodorov & Krasovsky 2019, Halley et al. 2021). At the time, it was believed that the North American and Eurasian beavers were a single species, as they are morphologically very similar (Morgan 1868). Four decades later, it was found that they have different chromosome counts (n = 40 and 48, respectively) (Lavrov & Orlov 1973) and cannot hybridise in nature (Parker et al. 2012). This situation has led to *C. canadensis* being considered an invasive alien species in Europe and Asia. There is great overlap in the biological and ecological niches with the native Eurasian beaver *C. fiber*, which could translate to competitive exclusion. Currently, the management actions for *C. canadensis* populations in Europe involve their removal (Parker et al. 2012, Halley et al. 2021).

Archaeological evidence has revealed that Eurasian beavers inhabited the middle and lower courses of the rivers in Bulgaria as early as 5,000 BCE (Boev & Spassov 2019). They are considered to have gone locally extinct in the 19th century (Beech 2007, Boev & Spassov 2019). In neighbouring Romania, the beavers disappeared by 1824, and they were reintroduced in the period between 1998 and 2003 along the Rivers Olt and Ialomița, from where they spread to other rivers (Ionescu et al. 2010), including the Danube Delta (Kiss et al. 2012, Bouroș et al. 2021). The first evidence of the return of beavers in Bulgaria was published at the beginning of 2021 (Kodzhabashev et al. 2021, Natchev et al. 2021), but the origin of these animals has remained unclear.

The long history of beaver translocations (including illegal; see Bertolino et al. 2023 and Dewas et al. 2012) and natural recolonisations throughout Europe (including *C. canadensis*) calls for genetic analysis of the newly established populations. Such studies have helped clarify the mechanisms of the species' spread across different countries (Kropf et al. 2013, Biedrzycka et al. 2014, Frosch et al. 2014, Senn et al. 2014, Minnig et al. 2016, Mai et al. 2018, Fedorca et al. 2021, Iso-Touru et al. 2021, Munclinger et al. 2022). Beavers are still rare in Bulgaria, and invasive genetic sampling would require considerable resources and frequent access to the habitat, which could disturb native fauna. Indeed, *in-situ* surveys are not always possible, especially in the case of the River Danube islands. The effectiveness of sampling beaver saliva from wood chips and using the obtained genetic material to distinguish between the two beaver species has been proven to garner high success (Iso-Touru et al. 2021). On the other hand, isolating semi-aquatic mammal DNA from their aquatic habitats has also been implemented in recent years (Padgett-Stewart et al. 2016, Sales et al. 2020, Burgher et al. 2024). Therefore, we aimed to apply these two non-invasive environmental DNA (eDNA) sampling techniques to determine whether the new beaver populations in Bulgaria belong to native or invasive species. This information will be used to develop appropriate management actions (e.g. eradication if *C. canadensis* is detected). Although eDNA is not commonly used to answer questions about intraspecific genetic diversity, we also attempted to acquire some initial insights about the geographical origin and genetic diversity of the sampled populations.

Material and Methods

Sites description

Five sites where our team and local collaborators have observed constant beaver presence since 2021 were sampled as part of this study. Three are located on the River Danube bank and the adjacent islands – Vardim, Belene, and Skomen. The other two sites are on the tributaries Yantra and Cherni Lom, located 22 km in a straight line (or 44 km along the river stream) and 19 km in a straight line (or 79 km along the river stream), respectively, from the Danube. The last two localities are in close proximity to human settlements and roads, while those along the Danube are more remote.

Sample collection

The sampling was done in September and October 2023 and February and April 2024. We collected two types of samples to target beaver DNA from the environment – water (n = 18) and wood chips (n = 29). The water samples (1 l each) were taken using sterile disposable gloves and containers. The water was filtered through nitrocellulose filters (d = 0.45 μm)

Fig. 1. Evidence of beaver activity in the field. The wood chips bitten off the branches and tree trunks were collected as samples. Photo Polina Nikova.

on-site using a manual pump within 2-3 hours after the sampling, according to the setting in Laramie et al. (2015). The filtering took 1-2 hours per sample on average. We collected two to five replicates from each site, targeting the vicinity of any signs of beaver presence (gnawed trees, tracks, feeding pathways, burrows) and downstream, wherever it was possible (up to 300 m) (Macher et al. 2021). We chose locations where the current was slow or absent, like channels, pools and swamps, never sampling the main course of the river. To avoid contamination, the pump parts that came into contact with water were changed or sterilised with a 50% bleach solution between samples. The filters were then stored in 95% ethanol, which proved practical and effective in the field (Laramie et al. 2015, Hinlo et al. 2017) and transported to the laboratory in a portable icebox. We surveyed the sites for gnawed trees and collected the freshest wood chips using sterile gloves (Fig. 1). The wood pieces were placed either in sterile tubes filled with 95% ethanol or in sterile plastic bags. The number of samples per site is presented in Table 1.

Table 1. Total number of samples collected and number of positive (i.e. successfully sequenced) samples (in parentheses) from the five known beaver localities in Bulgaria. Present haplotypes are given in parentheses after the site names.

Site (haplotype)	Skomen (fi1)	Pepelina (fi1)	Vardim (fi1)	Byala (ga1)	Belene (fi1)	Total	Success rate
Water samples	2 (2)	5 (0)	4 (0)	4 (0)	3 (0)	18 (2)	11%
Frozen wood chip samples	0 (0)	0 (0)	2 (0)	5 (1)	1 (1)	8 (2)	25%
Wood chip samples in 95% ethanol	3 (0)	1 (1)	7 (1)	4 (0)	6 (0)	21 (2)	10%
Hair samples	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	1 (1)	100%
Total	5 (2)	7 (2)	13 (1)	13 (1)	10 (1)	48 (7)	15%

In the lab, the ethanol samples were stored in a refrigerator (4 °C) and dry ones in a freezer (–18 °C), according to the methodology by Iso-Touru et al. (2021). In addition, one hair sample was also found and included in the study. It was stored in silica gel in a refrigerator (4 °C). Two tissue samples from wild Eurasian beaver populations in the south-eastern part of the Czech Republic were used as a positive control. These came from the Genetic Bank of the Institute of Vertebrate Biology of the Czech Academy of Sciences, which meets all legal requirements. One of them (№IVB-M-6709) was collected from roadkill under exemption no. JMK 13759/2016 from Act No. 114/1992 Coll., on Nature and Landscape Protection. The other (№IVB-M-9105) originated from an individual hunted legally under exemption no. KUJI 53537/2021 from Act No. 114/1992 Coll. Both these documents are available from the IVB Genetic Bank curator upon request.

DNA extraction

Beaver saliva from wood chips stored in ethanol was collected by rubbing a sterile cotton swab (Aptaca sterile swabs with a plastic stick, rayon tip) on the surface of the chips. The swabs were then placed in a sterile petri dish, and the ethanol was left to evaporate overnight. For dry-preserved wood chips, a drop of sterile water was applied on the swab tip to moisten it, and then it was rotated consistently over the wood chips to cover the maximum surface area (Iso-Touru et al. 2021). Afterwards, all swabs were placed into a 1.5 ml Eppendorf tube, and the shaft was trimmed to approximately 0.5 cm above the swab tip. The scissors used to trim the shaft were sterilised with alcohol and a flame between samples.

DNA from hair strands, swabs with saliva, and muscle tissue (positive control) was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. DNA from nitrocellulose filters was extracted via the DNeasy PowerWater Kit (Qiagen) according to the manufacturer's instructions. The tissue samples, the hair and the environmental samples were processed separately to avoid cross-contamination. Other measures against contamination were also taken during the extraction process, such as using sterile gloves, filter tips and negative controls.

PCR amplification and sequencing

Total DNA was used as a template for the subsequent polymerase chain reaction (PCR). The PCR was conducted with the HotStarTaq Plus Master Mix Kit (Qiagen) according to the manufacturer's requirements. A mitochondrial fragment of approximately 800 bp, including the D-loop, was amplified with the primer pair used by Iso-Touru et al. (2021) (F 5′-TCCACACATCAAAACAACGAA-3′ and R 5′-GGCCCTGAAGTAAGAACCAG-3′). For every PCR, nuclease-free water was used as a negative control and muscle tissue DNA was used as a positive control. The following temperature regime was used: 95 °C for 5 min, 40 cycles of 30 sec denaturation (94 °C), 30 sec annealing (58 °C), and 30 sec elongation (72 °C), and a final 10 min elongation (72 °C). Successfully amplified samples were sequenced using the Sanger method in both directions by Macrogen Europe BV. Obtained sequences were edited with CodonCode Aligner v.8.0.2 (CodonCode, Dedham, MA, USA).

Molecular identification and phylogenetic analyses

Each newly obtained DNA sequence was initially used to query the NCBI core nucleotide database via the BLAST algorithm (Madden 2002). The matching sequences of the mitochondrial D-loop of the Eurasian beaver were downloaded from GenBank. DNA alignments were created with MEGA X using the MUSCLE algorithm (Kumar et al. 2018). A mitochondrial haplotype network was created using the Median-Joining algorithm (Bandelt et al. 1999), implemented in PopART v. 1.7. (Leigh & Bryant 2015), adding the new data to the previously described European beaver haplotypes (Durka et al. 2005, Horn et al. 2010, Kropf et al. 2013, Frosh et al. 2014, Iso-Touru et al. 2021, Munclinger et al. 2022).

Results

DNA extraction success rate

Seven mitochondrial D-loop sequences (approximately 800 bp) were obtained (GenBank accession numbers PP825818-PP825824) (Table 2) from a total of 29 wood chip samples, 18 water filtrate samples and a single hair sample processed (Table 1). Extracting beaver mitochondrial DNA from the water filters was less successful (11%, two out of 18) than from the saliva on gnawed wood chip pieces. Preserving wood chip samples frozen proved more fruitful (25%, two out of eight) than placing them in ethanol (10%, two out of 21). A sequence was also obtained from the single hair sample.

Molecular identification and phylogenetic analyses

All sequences showed a 100% match with published mtDNA D-loop sequences of the Eurasian beaver in

the BLAST Tool (NCBI 2024). Two distinct haplotypes were observed in Bulgaria – sequences BUL1-5 and BUL7, found in four locations (Skomen, Pepelina, Vardim and Belene), belong to the haplotype fi1 (Durka et al. 2005) (identical to haplotype CFIB1 in Iso-Touru et al. 2021), while sequence BUL6, collected near the town of Byala only (Figs. 2, 3), was identical with haplotype ga1 (Durka et al. 2005).

Discussion

The Eurasian beaver experienced a major population bottleneck in the 18th and 19th centuries, with only a few countries retaining relict populations, and Bulgaria was not among them (Halley et al. 2021). Since 2021, there have been reports of beavers returning to Bulgaria after an absence of over 150

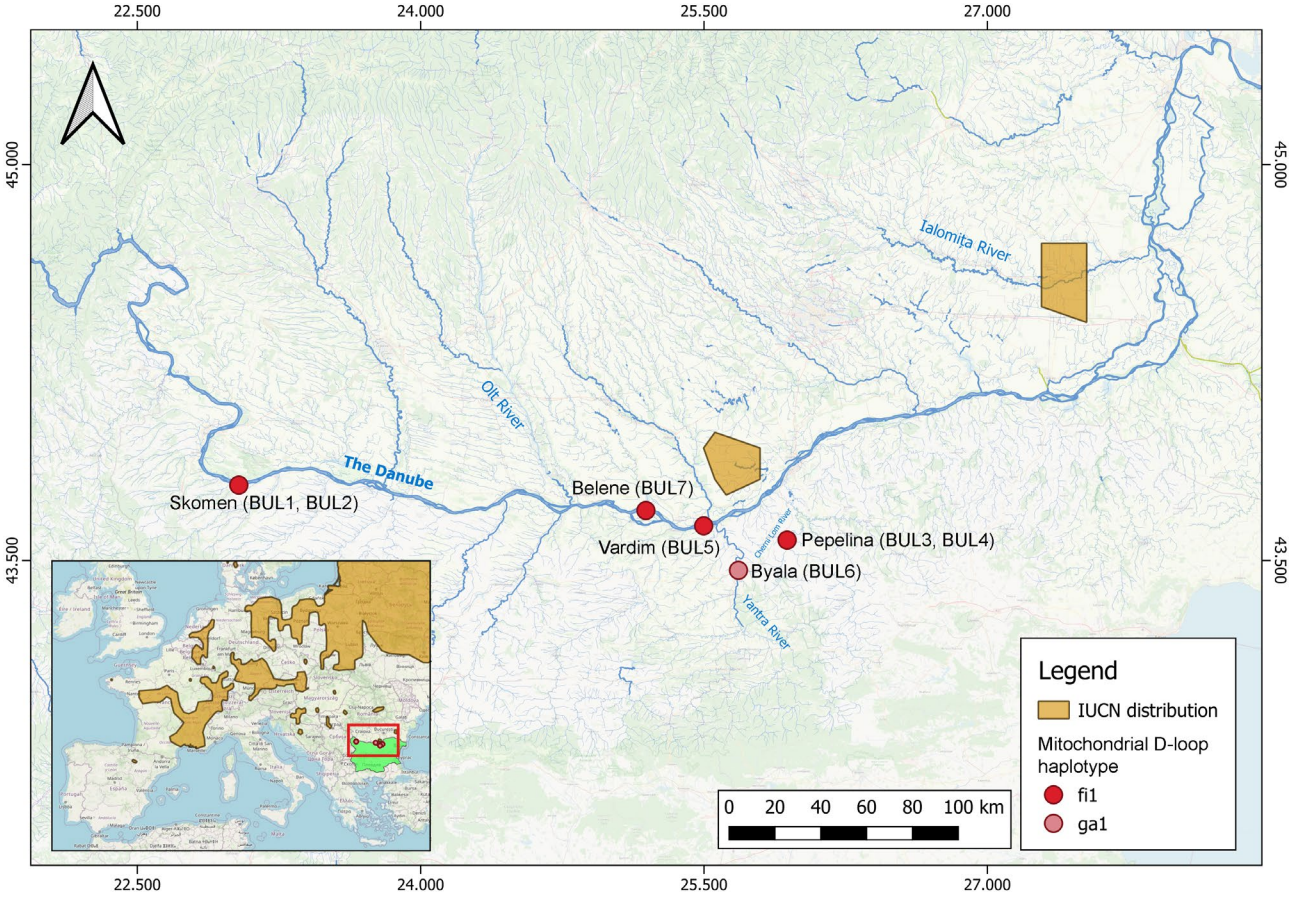

Fig. 2. Map of sampled localities of beavers in Bulgaria (circles). The haplotype identity of each sample is shown in different shades of red. A map of Europe is inset, where Bulgaria is marked in green, and the study area is highlighted with a red frame. The current distribution of the Eurasian beaver (*C. fiber*) according to the IUCN is shown in beige (Batbold et al. 2021). The sequence IDs corresponding to the particular localities are given in parentheses (Table 2). The map was created using QGIS version 3.34.2-Prizren and is available at https://www.qgis.org/. The EU Danube River Net is a Copernicus Land Monitoring Service product that is funded by the European Union (https://doi.org/10.2909/393359a7-7ebd-4a52-80ac-1a18d5f3db9c). Base map © OpenStreetMap contributors (CC-BY-SA) are available at https://opentopomap.org/.

Table 2. Mitochondrial D-loop haplotypes and their GenBank accession numbers in beaver localities in Bulgaria.

Sequences ID (Accession No)	Locality	Haplotype (Durka et al. 2005)	Latitude	Longitude
BUL1 (PP825818)	Skomen	fi1	43.7889	23.0374
BUL2 (PP825819)	Skomen	fi1	43.7889	23.0374
BUL3 (PP825820)	Pepelina	fi1	43.578	25.9404
BUL4 (PP825821)	Pepelina	fi1	43.5785	25.9404
BUL5 (PP825822)	Vardim	fi1	43.6331	25.4985
BUL6 (PP825823)	Byala	ga1	43.4625	25.6827
BUL7 (PP825824)	Belene	fi1	43.6919	25.1927

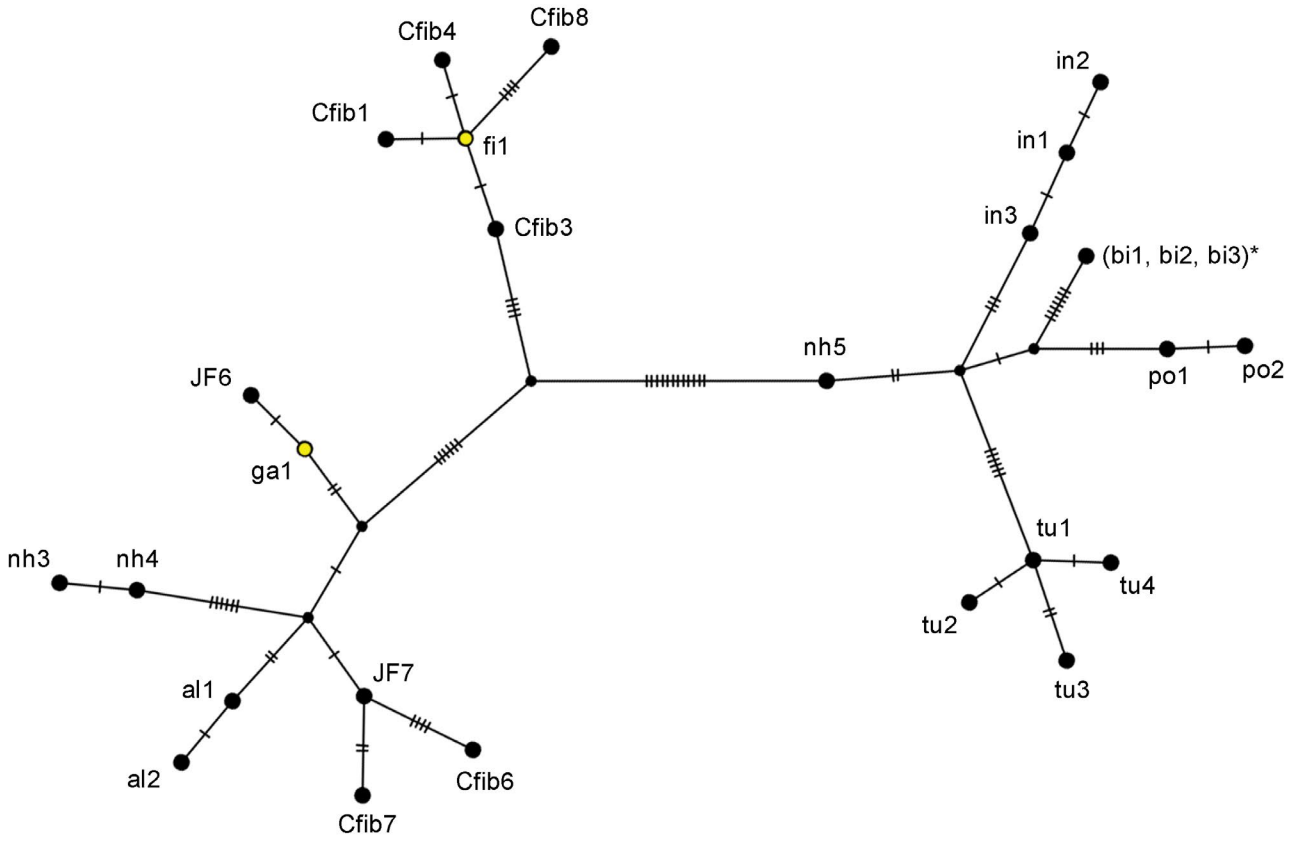

Fig. 3. Median-joining haplotype network of European *C. fiber* populations, based on a 484 bp fragment of the mtDNA D-loop. Haplotypes found in Bulgaria are indicated in yellow. *Haplotypes bi1, bi2 and bi3 differ only in indels.

years (Kodzhabashev et al. 2021, Natchev et al. 2021), but the origin of the individuals was not clear. Using genetic data gathered from the environment in the form of gnawed wood, water samples and hair from five locations, we infer that Bulgaria is now home to individuals from the species *C. fiber*, carrying haplotypes that originate from the French and Norwegian refugial populations (Durka et al. 2005, Senn et al. 2014, Munclinger et al. 2022). Below, we discuss the methodology used for the sampling and genetic analysis, as well as several hypotheses for the origin of Bulgarian beavers based on our results.

Methodological considerations

Our results suggest that isolating DNA from the saliva on gnawed wood is an effective way to identify the species and haplotypes in the southern part of the Eurasian beaver's range, as it was reported for its northern part (Iso-Touru et al. 2021). The sequencing results showed no contamination problems, so the anti-contamination measures we took can be considered sufficient. We recommend taking multiple samples from each location to maximise the chances for obtaining high-quality DNA. However, the success rate was considerably lower in our study (25% for frozen samples *vs.* 83% in Iso-Touru et al. 2021). This discrepancy could be due to differences in the methodology, such as using different swabs and having an additional step of overnight DNA precipitation of the samples with sodium acetate and ethanol that were applied in Iso-Touru et al. (2021). Another factor impacting DNA extraction could be the higher temperature and the solar irradiation in southern Europe, compared to Finland, so sampling in winter and early in the morning could be more effective. As for the storage methods, the frozen samples gave better results than the ethanol samples (25% *vs.* 10% successfully sequenced, respectively). One possible explanation for this difference could be that the DNA was washed into the ethanol and precipitated to the bottom of the tube. Processing the solution could improve the results.

Water sampling for DNA extraction has been applied recently to survey semi-aquatic mammals (Padgett-Stewart et al. 2016, Sales et al. 2020, Burgher et al. 2024). However, in the current study, the success rate of DNA extraction from filtered water samples was low – 11% (compared to 57% in Sales et al. 2020 and 87% in Burgher et al. 2024). We chose sampling locations where the current was weak or absent to avoid DNA dilution, though that could have led to higher degradation rates due to the increased temperature and presence of bacteria in stagnant

waters. This challenge could be overcome if shorter DNA fragments are amplified (Takahashi et al. 2013), but these would be less informative. Although there are advantages to collecting a small amount of water, including faster filtering, which leads to quicker preservation of the samples, it could still be more beneficial to sample a larger volume of water, as that might increase the content of DNA in the samples (Capo et al. 2019). Yet, extracting DNA from gnawed wood using a conventional kit was more successful, cheaper, simpler and faster, as no filtering or special tools were required, as opposed to working with water samples. The only necessary equipment is sterile gloves, containers and an icebox, which makes it easier to combine with other classical monitoring routines like transects and citizen science (Iso-Touru et al. 2021).

Lastly, we consider extracting DNA from hair follicles as another promising method for obtaining genetic material that has already been successfully applied in other studies (Herr & Schley 2009, Frosch et al. 2014, Sobkowiak et al. 2021). The only drawback is that, according to our experience, it is rare to find hair by chance when visiting a beaver habitat. To increase the probability, placement of hair snares along active beaver paths is needed, and in relation to this, a second field visit after a certain period is required. This approach requires more time and effort than the two eDNA methods.

Possible origin of the beavers in Bulgaria

Bertolino et al. (2023) proposed four hypotheses for the origin of newly found beaver populations in Italy, which could also be considered in the case of Bulgaria: 1) cryptic residual populations, 2) natural dispersal, 3) escape from captivity, or 4) illegal (undocumented) release. Four of the five sampled locations in our study revealed the presence of one single mitochondrial haplotype, which points to a single source of recolonisation despite the distance between the locations (approximately 250 km in a straight line). This finding suggests that a small number of individuals of relatively close ancestry entered the country recently, either through dispersal or introduction. The most parsimonious explanation for the origin of the contemporary beaver populations in Bulgaria is that they naturally spread southward from Romania. The geographically closest sources are the expanding populations along the Rivers Olt and Ialomița in Romania, which were reintroduced from Bavaria between 1998 and 2003 (Ionescu et al. 2010, Fedorca et al. 2021). The German populations were established in the second half of the 20th century by importing individuals from refugial populations in France, Norway, Russia and Poland (Zahner 1997, Frosch et al. 2014, Mai et al. 2018). After releasing 35 individuals in the area near Slobozia in 2003 along the River Ialomița (Ionescu et al. 2010), their presence was reported at Parcheș in 2009 and at Maliuc in 2011, the distance from Slobozia to Maliuc being approximately 150 km in a straight line (C. Pașca, pers. comm.). Furthermore, several individuals have been reported in the Danube Delta since 2011 (Kiss et al. 2012, C. Pașca, pers. comm.). In the River Olt, 91 individuals were released between 1998-2001 (Ionescu et al. 2010). However, probably due to the high number of hydropower dams along the river, they reached the Danube at a much slower pace compared to the ones in Ialomița (the distance from Făgăraș, near where they were released, to River Olt's mouth in the Danube is > 200 km) (C. Pașca, pers. comm.).

The fact that the beavers sampled within our study possess mitochondrial haplotypes originating in the known relict populations in Norway and France fails to support the cryptic population hypothesis. Furthermore, beaver presence is easily detectable (e.g. fallen or gnawed trees) and could not have remained hidden for centuries in northern Bulgaria's highly antropogenically impacted river valleys. Escape from captivity is unlikely as we are not aware of any beavers being kept in local zoos or other public collections. On the other hand, illegal/undocumented release cannot be completely ruled out, as such actions have been suspected on several occasions in Europe (Dewas et al. 2012, Bertolino et al. 2023). There are records of beavers that have been released in the past for the enrichment of local game fauna in the eastern part of Bulgaria (Dragoev 1978), but we have no information on recent initiatives of this kind. Similar unregulated actions can have dire consequences for local ecosystems and people, as they lack consideration of the socio-economic context (Carver et al. 2021, Drouilly & O'Riain 2021). Beavers may bring benefits to freshwater habitats and reduce flood risk (Neumayer et al. 2020, Larsen et al. 2021, Auster et al. 2022), but under certain circumstances, they may increase that risk (Larsen et al. 2021), impede agricultural activities and thus initiate human-wildlife conflicts (Campbell-Palmer et al. 2016), restrict fish migration and spawning (Kemp et al. 2012), or negatively impact vegetation (Rosell et al. 2005, Mikulka et al. 2022b), as well as wildlife via zoonoses (Girling et al. 2019). These can lead to a negative perception of beavers among the relevant stakeholders (Hohm et al. 2024). Well-

informed management actions could mitigate potential conflicts and avoid obstruction of the environment (Larsen et al. 2021). In Bulgaria, the newly established small populations are especially vulnerable if such human-wildlife conflicts arise, e.g. beavers damaging the commercial poplar plantations near rivers. Simple mitigation measures like increasing the distance between the river bank and the plantations to 10-20 m could help, as most of the beaver activity is concentrated within this perimeter (Mikulka et al. 2022a). In addition, other soft-wooded species like *Salix* sp., which beavers prefer, could be planted between the river bank and the commercial plantations (Mikulka et al. 2022a). Apart from such direct conflicts, beavers could suffer from unintentional killing, namely drowning in stationary fishing nets (Kiss et al. 2014, C. Pașca, pers. comm.). An awareness campaign among fishermen could motivate them to avoid core beaver territories or reduce the duration of net and basket-trap submersion in the water to mitigate this threat.

Future perspectives

Contemporary habitat suitability models on a European scale show that Bulgaria possesses habitats of moderately high suitability based on climatic and habitat characteristics (Falaschi et al. 2024). Apart from the return of the Eurasian beaver in the north, the reintroduction of the animal is planned in Greece (Galanaki et al. 2022), which could later expand in the catchment area of the River Mesta (Nestos) in southwestern Bulgaria. This presents an opportunity for the species to successfully recolonise a large part of the country in the coming decades, as has already happened in other European countries like the Czech Republic (Barták et al. 2013) and Hungary (Czabán & Juhász 2024). Currently, *C. fiber* is officially declared extinct in Bulgaria and is thus not legally protected. In order to secure this positive conservation trend, the most important step is the inclusion of the species in the Biodiversity Act as strictly protected (Annex 3) and as a species requiring the designation of Natura 2000 sites (Annex 2). We suggest this will be amended swiftly, as the species is listed under the Bern Convention (Appendix III) and the EU Habitats and Species Directive (92/43/EEC). We hope that the genetic identification of the observed populations presented here will trigger this process and that methodological considerations will contribute to developing a monitoring protocol.

Acknowledgements

*We greatly acknowledge Radoslav Yordanov and the kayak club 'Svishtov' for their assistance in the fieldwork. We thank Milcho Todorov (IBER-BAS) for providing data on the beavers' occurrence on Skomen Island. We are grateful to Claudiu Pașca for providing information on the current and historical distribution of beavers in Romania. We also acknowledge the four anonymous reviewers and the editor, who helped us to improve our manuscript considerably. Beaver tissue samples were provided by the Genetic Bank of the Institute of Vertebrate Biology, Czech Academy of Sciences (part of the National Animal Genetic Bank, Czech Republic). This study was funded by the Bulgarian National Science Fund, grant contract KP-06-M61/1 of 13.12.2022, 'Return or Invasion? Study of the distribution and ecological patterns of beaver (*Castor fiber*) in Bulgaria after more than 150 years of absence'. B. Rolečková was financially supported by the Institutional Research Plan RVO: 68081766. The authors have no relevant financial or non-financial interests to disclose.*

Author Contributions

All authors contributed to the conception and design of the study. Material collection was performed by P. Nikova, M. Kachamakova, V. Todorov, B. Dimitrova, Y. Koshev, M. Ignatov, J. Uhlíková and B. Rolečková. Genetic analysis was performed by A. Bobeva and P. Nikova. Phylogenetic analysis was conducted by S. Borissov. The first draft of the manuscript was written by M. Kachamakova, P. Nikova, A. Bobeva and S. Borissov, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

The new sequences generated in this study are publicly available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/, accession numbers PP825818-PP825824).

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