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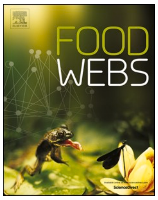

Full length article

A geometric morphometric approach to identify uncomplete snake vertebrae from raptor bird feeding remains

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ABSTRACT

The Hungarian meadow viper (*Vipera ursinii rakosiensis*) is an endangered subspecies of *Vipera ursinii*, which faces high predation pressure, partially due to avian species. To create a systematic method for estimating the measure of predation pressure, we developed a geometric morphometric approach to identify both undamaged and damaged vertebrae of snake species found in Hungarian meadow viper habitats from raptor feeding remains. We used linear discriminant analysis with a reference material of vertebrae from identified snake species as training data. We also tested its efficiency by predicting the identification results of different simulation levels based on vertebra completeness. We practiced this method on vertebrae of unknown species of snakes obtained from nests and pellets of short-toed snake eagles (*Circaetus gallicus*, $n = 9$), common buzzards (*Buteo buteo*, $n = 14$) and Montagu's harriers (*Circus pygargus*, $n = 3$). The identification approach showed high accuracy, even in the case of missing landmarks to some extent. We identified vertebrae remnants of *Natrix natrix* ($n = 172$, 83.9%), *Coronella austriaca* ($n = 10$, 4.9%) and *V. u. rakosiensis* ($n = 23$, 11.2%). Both, the reptile specialist *C. gallicus* and the generalist *B. buteo* proved to be preying on *V. u. rakosiensis*, while samples of *C. pygargus* did not contain any snake remains despite of previous observations of *V. ursinii* predations. Our approach is applicable for other studies and taxa as well, therefore can be a practical tool for classification of incomplete vertebrae, which is otherwise hardly identifiable. Furthermore, it could be applied to help estimate predation pressure on endangered snake species.

1. Introduction

Reptiles are one of our planet's most endangered animal groups partly due to their small home ranges and thermoregulatory constraints (Böhm et al., 2013). Besides threats such as human activity, habitat loss and degradation or climate change, predation by invasive predators is also significant (Gibbons et al., 2000). For already declining populations of endangered reptile species, natural threats like abundant native predators can further aggravate their chances of survival. Avian predators that primarily hunt visually can exploit the vulnerability of snakes and lizards during thermoregulation (Webb and Whiting, 2005; Anderson and Burgin, 2008). They commonly prey upon snakes, as species of at least 12 avian orders have been recorded as snake predators, and several species hunt them regularly (Martin and López, 1990; Tanaka and Mori, 2000; Neal and Steen, 2015). Some of them even specialize in hunting reptiles, such as snake eagles (*Circaetus* spp.).

Generalist predators that exploit the absence of their own predators (mesopredator release hypothesis) or certain anthropogenic resources can grow tremendously in population size, hence they could threaten the populations of a wide range of taxa, even if they are native to their ecosystem (Boarman, 2003; Ripple et al., 2013; Read and Scoleri, 2015). For instance, raptors are keen to use artificial perches placed in

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agricultural or grassland habitats for hunting. and prefer such habitats over ones that lack sufficient perching opportunities (Hall et al., 1981; Widen, 1994; Zagorski and Swihart, 2020). Controlling the threat posed by invasive or native predators is often needed to conserve already vulnerable prey (Reardon et al., 2012; Móré et al., 2022). Consequently, it is fundamental to determine the measure of predation (Sinclair et al., 1998). There are numerous methods for this, with varying efficiency. Direct observation of predator species is suitable for activity estimation, but it requires substantial working capacity, while observations of predation are scarce. Estimating abundance of certain predators with camera trapping surveys can be a reliable method to assess population size (Burton et al., 2015), but it does not necessarily reflect the prevalence of predation on given prey species. Dietary studies on the other hand give information about both the predator and the prey (Zanette et al., 2003).

Identification of prey remains is often challenging. Molecular methods such as DNA barcoding (Zeale et al., 2011) and metagenomic sequencing (Paula et al., 2015) are generally more successful at identifying prey species in predator diets than traditional morphological methods but can be considerably more expensive and infrastructure demanding (Mumma et al., 2016). Snake vertebrae have multiple species-specific morphological traits and are snakes' easiest parts to recover from predators' dietary remnants due to their great numbers in one specimen and their moderate sturdiness (Ratnikov, 2004; Ikeda, 2007). This makes them appropriate objects to assess predator-prey interactions.

Morphological traits can be described by visual evaluation, but it is time-consuming and requires an expert, whereas morphometric methods prove to be more objective and bear replicable results (Mutanen and Pretorius, 2007). Morphometric methods work with multivariate calculations, but while traditional morphometry uses distances and captures an object's size, geometric morphometry uses coordinate data and captures shape (Mutanen and Pretorius, 2007). Geometric morphometry became a popular method in numerous fields of biology (Adams et al., 2004), for instance in herpetology where it is applied to study adaptive shape patterns, phenotypic plasticity, and sexual dimorphism among other things (Kaliontzopoulou, 2011). It is an effective, cheap, and objective method for species identification, and can be even more accurate than DNA barcoding in certain cases (Ibañez et al., 2007; Sauer et al., 2020; Chaiphongpachara et al., 2022). The geometric morphometric assessment of snake vertebrae has been successfully applied in a few studies before, for instance to prove the presence of different vertebral regions in snake-like forms (Head and Polly, 2015).

The Hungarian meadow viper (*Vipera ursinii rakosiensis,* Méhely 1893) is an endangered, grassland dweller subspecies of *Vipera ursinii* (Bonaparte 1835). Despite decades of conservation efforts, there is moderate or no detectable increase in its abundance, most likely due to habitat degradation and high predation pressure (Móré et al., 2022). In this study, we developed a geometric morphometric identification method for snake species in habitats of *V. u. rakosiensis* that is fit to analyze partial vertebrae as well, not just intact specimens. We show the efficiency of this novel approach in specific identification of incomplete vertebrae through simulations. We also present the results of identification of snake vertebrae recovered from nest material and pellet samples collected from and around nests of short-toed snake eagles (*Circaetus gallicus,* Gmelin 1788), common buzzards (*Buteo buteo,* Linnaeus 1758) and Montagu's harriers (*Circus pygargus,* Linnaeus 1758) in, or in the proximity of *V. u. rakosiensis* habitats in Hungary, Kiskunság.

2. Methods

2.1. Study area

We collected samples at the two largest known *V. u. rakosiensis* habitats in Hungary, "Felső-kiskunsági turjánvidék" HUKN20003 Natura 2000 site (from now on Peszéradacs) and "Bócsa-bugaci homokpuszta" HUKN20024 Natura 2000 site (from now on Bugac) and their periphery (Fig. 1). The Peszéradacs site is a network of grasslands of various structures and types spread over more than 5000 ha. The site consists of dry sandy grasslands, mesophile grasslands and also marsh meadows. The Bugac site, unlike the Peszéradacs, mostly consists of dry or mesophile sandy grasslands, but a small amount of marsh meadows are also present in the area. This site is spread over more than 11,000 ha. At both sites forests, forest edges and tree rows are typical alongside the open grassland habitats, which are ideal nesting and perching places for birds, but additional, artificial perches can be found directly in the habitats as well, such as electric wire fences used for controlled grazing, or wells.

2.2. Study species

2.2.1. Predator species

Common buzzard (*Buteo buteo)* is present in large numbers and is a known predator of *V. u. rakosiensis*. They are generalists with a hunting area of one to two km^2 during nesting season rearing two to three chicks (Haraszthy, 2022).

Montagu's harrier (*Circus pygargus*) is also a frequent species at the study sites and has also been observed while carrying a deceased *V. u. rakosiensis* specimen. They are generalist and opportunistic predators that are known to prey on reptiles, though mainly on lizards and only on rare occasions on snakes (Arroyo, 1997). Their hunting area during nesting season is 8–17 km^2, usually with a clutch size of two to five eggs, more accurately 1,9–3,5 fledglings per successful nest (Turny et al., 2022).

The short-toed snake eagle (*Circaetus gallicus)* is a snake specialist predator (Papp et al., 2022). Several specimens have nests close to the viper habitats and frequently forage at those, but they are rare compared to *B. buteo* or *C. pygargus*. They also hunt in a bigger area, usually 16–30 km^2 but sometimes even 100 km^2, and have a clutch size of one chick (Papp et al., 2022).

2.2.2. Prey species

The Hungarian meadow viper (*Vipera ursinii rakosiensis*) is a grassland specialist species, one of Europe's most endangered vertebrates (Korsós, 1992). It is a relatively small, 40–50 cm long, bulky snake. It has a base colour of light grey or yellowish brown and a dark zigzag pattern on its back, which, according to some, is an aposematic feature in related species (Niskanen and Mappes, 2005; Wüster et al., 2004). Historically it was common in the Vienna Basin, around Lake Fertő, the Hanság region, around Budapest, in the Kiskunság region, and the surroundings of Cluj-Napoca in the Transylvanian Plain (Mizsei et al., 2018; Móré et al., 2022). Along with agricultural intensification, most of its habitats were converted to arable croplands, thus the Hungarian meadow viper's habitat range shrunk drastically, moreover, the remaining populations also suffered a significant decline due to the intensified grassland utilisation and excessive predation pressure (Péchy et al., 2015; Móré et al., 2022). Recently only a few small, isolated populations remain in the Hanság, Kiskunság and Transylvanian Plain areas (Mizsei et al., 2018). Thanks to the continuously ongoing, specific LIFE projects since 2004, the habitats had been extended and national parks implemented viper-friendly grassland management in several areas (Mizsei et al., 2020). In addition, declining populations were reinforced with individuals from ex situ breeding or established new populations (Péchy et al., 2015; Mizsei et al., 2020). In spite of the conservation efforts of the last decades, its global population size did not grow significantly, and one of the most important reasons for that is predation pressure (Móré et al., 2022). The Hungarian meadow viper is mostly preyed upon by native predators, most importantly the European badger (*Meles meles*), the red fox (*Vulpes vulpes*) and several avian predators (Móré et al., 2022).

Besides *V. u. rakosiensis*, grass snakes (*Natrix natrix*, Laurenti 1768) and smooth snakes (*Coronella austriaca*, Laurenti 1768) are present in

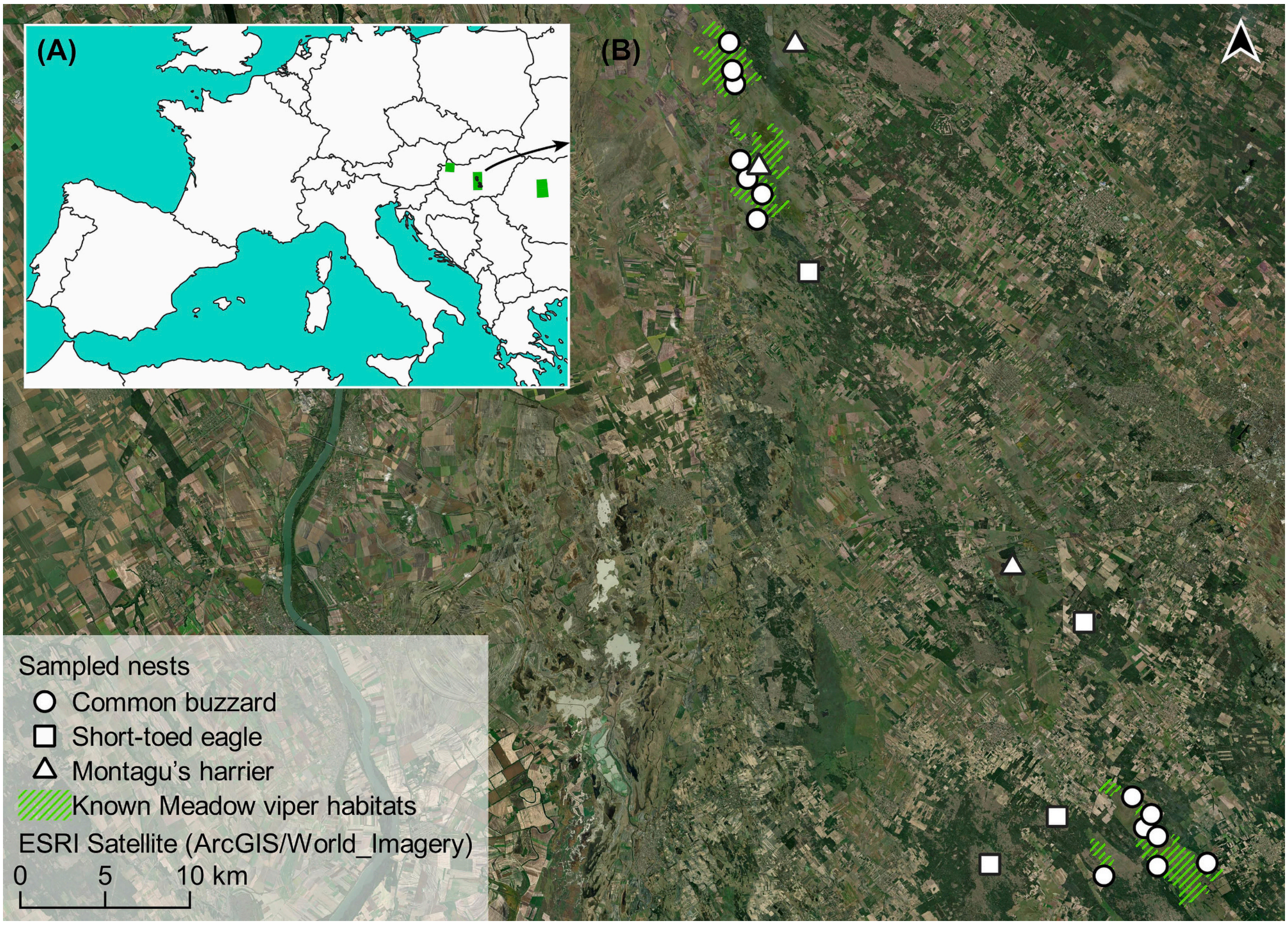

Fig. 1. Sampled nests of studied avian predators and known Hungarian meadow viper habitats in the study site, Kiskunság.

both habitats. *N. natrix* is larger, 100–120 cm long, but a thin snake compared to *V. u. rakosiensis*. Their base colour can be quite variable, mostly a shade of grey, and they usually have a yellow-black bordered collar, which also might be an aposematic feature in juvenile individuals (Madsen, 1987). They prefer mostly wetland habitats, however, they occur in many different types as well, even in sandy grasslands where they mostly prey on small rodents (Gregory and Isaac, 2004; Hojati et al., 2012).

C. austriaca is a quite small, 60–80 cm long, and thin snake. Its coloration is similiar to that of *V. u. rakosiensis* but has a milder pattern. It occurs in a variety of mostly open habitat types, where its main food source, lizards are abundant. *C. austriaca* is a reptile specialist predator (Brown et al., 2014). It mostly feeds on lizards, but it is known for its frequent snake (Drobenkov, 2014) and occasional viper predation (Capizzi et al., 1995; Luiselli et al., 1996; Pardavila et al., 2012; Groen, 2018; Di Nicola et al., 2020).

2.3. *Sample collection and processing*

In the case of *Buteo buteo*, during wintertime we located the nests when the missing canopy foliage helped the detection. As *Circaetus gallicus* nest on pine trees, to locate nests we did visual observation during breeding time and located the nest at the second half of the feeding period. We located *Circus pygargus* nests during the breeding period by visual observation of the behaviour of parent birds. During the nesting time, we checked the nests several times for the presence of breeding birds. We collected nest samples and pellet samples during the ringing of the chicks for other conservation purposes or when the nest became abandoned (Fig. 1).

We subsequently processed the collected nest and pellet material by extraction of all bone remains from the samples, then sorted out and cleaned every snake vertebra based on the morphological descriptions in Venczel (2000).

2.4. *Vertebrae identification*

We developed a novel approach to identify snake vertebrae based on linear discriminant analysis (LDA) in a geometric morphometric framework fitted on landmarks of training vertebrae with known species identity and subsequently predict group membership probability on the samples with unknown species identity. As vertebrae extracted from predator nests and pellets are often damaged, we developed the identification process to remove the missing landmarks from the training dataset as well before the LDA is fitted. During the identification process, for each LDA a confusion matrix is created, and at the end of the run, we acquire the group membership probability for the unknown samples (species identity), as well as the specificity and sensitivity of the LDA responsible for identification.

2.5. *Geometric morphometric landmarks*

To identify unknown vertebrae, firstly we created a landmark database on known specimens (training dataset) and unknown vertebrae, obtained from nest and pellet samples (unknown dataset). For this, we had taken focus stack macro photographs (Nikon D600, Micro-Nikkor 55 mm f2.8, in manual mode 1/200, f13 using Nikon R1C1 Dual SB-R200 speedlight) of the ventral sides of 939 vertebrae, from the collection of the Hungarian Natural History Museum (*V. u. rakosiensis*: $n = 392$, specimens MD4/51d/C, MD4/52d/C, MD4/56d/C, MD4/88d/C; *N. natrix*: $n = 471$, specimens MD4/29d/C, MD4/30d/C, MD4/31d/C, MD4/35d; *C. austriaca*: $n = 76$, specimen MD4/12d/C). Image stacks were produced in the software CombineZP (Hadley, 2010).

We handled presacral and caudal vertebrae separately in all three species since they have fundamental morphological differences. We determined 15 landmarks for presacral and 16 landmarks for caudal vertebrae (Fig. 2), and retrieved their coordinates for each vertebra, using the software ImageJ 1.53e (Schneider et al., 2012).

2.6. Identification process

1. *Merging and Creating Subsets by Vertebra Type*: We merged the training and unknown landmark datasets, created subsets based on vertebra type (presacral, caudal). This distinction is crucial as the shape and number of landmarks differ between these types, necessitating separate analyses for accurate identification.
2. *Handling Landmark Missingness*: In Step 2, we addressed the issue of incomplete specimens, a common challenge in field-collected bone samples. After categorizing the completeness of each vertebrae, we created subsets for each pattern of landmark missingness. Critically, for subsets with missing landmarks, we also removed these landmarks from the training dataset. This step ensures consistency between the training and unknown datasets, allowing for a more accurate analysis. It essentially means that if a landmark is missing in the unknown set, it is also excluded from the training set to maintain comparability.
3. *Standardization Through Generalized Procrustes Analysis (GPA)*: For the subsets, we applied GPA to standardize landmark coordinates using the gpagen function of the geomorph package (Baken et al., 2021; Adams et al., 2022). In morphometric analyses, raw coordinates are influenced by size, position, and orientation, which can obscure the shape information we are interested in. GPA removes these non-shape variations, aligning all specimens in a common coordinate system. This process allows us to compare the shapes of the samples directly, which is essential for our subsequent analyses. Without this standardization, any differences we observe might be due to irrelevant factors like size or orientation rather than genuine shape differences.
4. *Fitting LDA and Predicting Group Membership*: We utilized Linear Discriminant Analysis (LDA) to analyze the standardized dataset. LDA helps in distinguishing between different groups (snake species, in our case) based on the landmarks. The lda function from the MASS package (Venables and Ripley, 2002) was used for training, and the predict function from the stats package (R Core Team, 2023) helped in assigning group membership probabilities to unknown samples.
5. *Evaluating LDA Performance*: Finally, we used cross-tabulation and the confusionMatrix function from the caret package (Kuhn, 2008) to assess the sensitivity and specificity of the LDA model. This evaluation is important to understand the accuracy of our model in correctly classifying the vertebrae types.

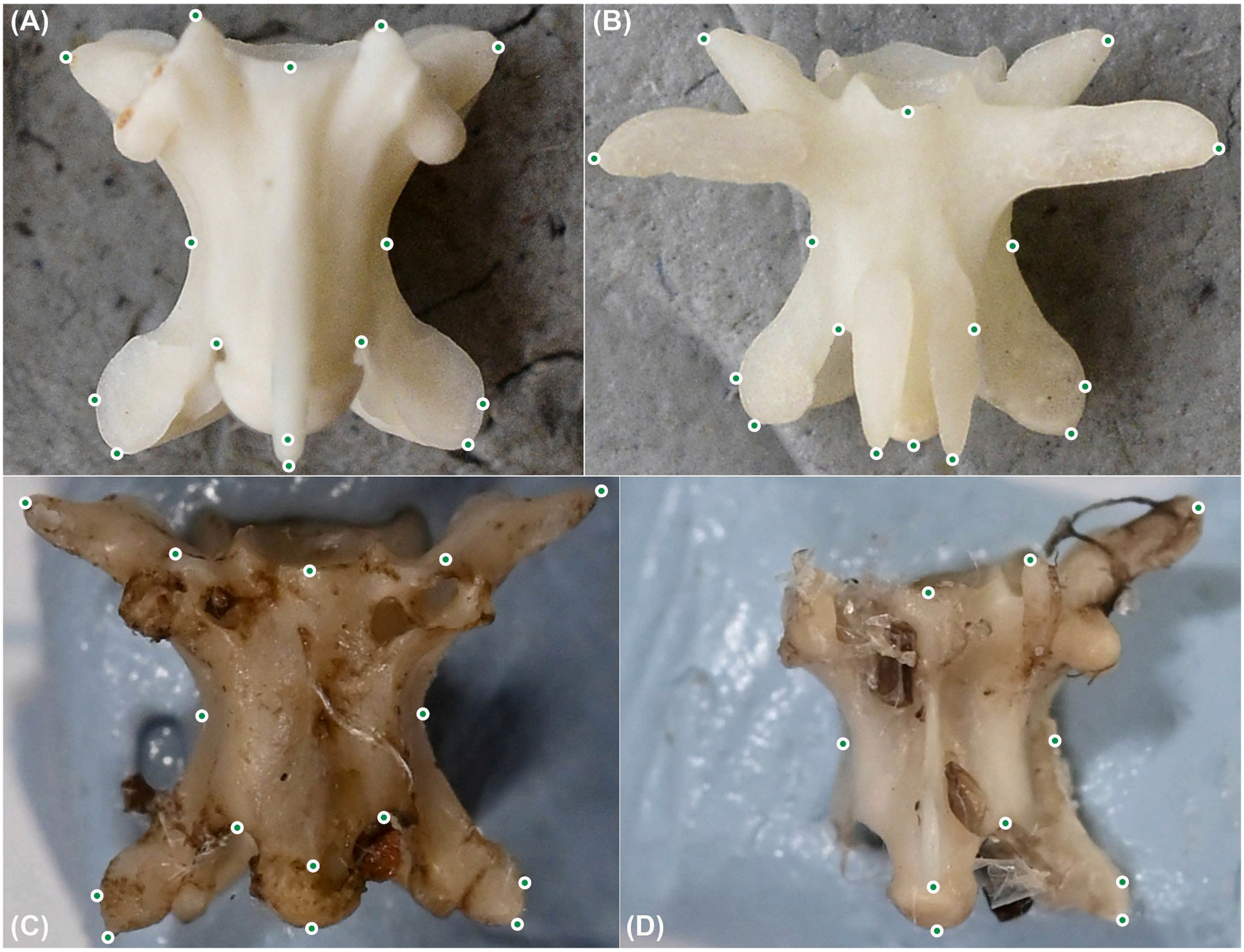

Fig. 2. Used landmarks on reference (A, B) and collected (C, D), presacral (A, C, D) and caudal vertebrae (B).

2.7. Simulation to test efficiency

To assess the efficiency of the above-described identification method, we randomly generated datasets from the training samples, set to be unknown samples with missing landmarks, and ran 100 rounds of simulation for missingness levels 0–75% with 5% steps. The identification process was the same in the simulation as described above, except the confusion matrix was created by cross-tabulation of the real identity of the training samples handled as unknown samples and predicted their identity.

We performed all steps of data processing and statistical analyses in R 4.1.3 (R Core Team, 2023).

3. Results

3.1. Geometric morphometric differences of the studied prey species

According to the LDA, vertebrae of each species have geometric morphometric differences (Fig. 3). Regarding presacral vertebrae, the separation of species is more defined, only *V. u. rakosiensis* and *N. natrix* have a slight overlap. Caudal vertebrae of all three species have more considerable overlap, and the points are not as cohesive, hence caudal vertebrae have larger morphological variance.

3.2. Simulation results

Regarding presacral vertebrae, sensitivity and specificity of identification is at least 90% at 75% of vertebra completeness (12 out of 16 landmarks) or higher, in all three species (Fig. 4). From 50% of vertebra completeness or lower, all three values start to heavily decrease (especially sensitivity of *C. austriaca*), except for specificity of *C. austriaca*, which remains near 100%. While sensitivity is slightly higher in *V. u. rakosiensis* than in *N. natrix*, the reciprocal is true for specificity. *C. austriaca* visibly had larger deviations in different simulations.

In the case of caudal vertebrae, values were more dispersed (Fig. 4). Both sensitivity and specificity are between 90% and 100% at 100% of vertebra completeness for all three species. Values for *V. u. rakosiensis* and *N. natrix* decrease at a quicker rate with lower vertebra completeness than presacral vertebrae do, except for the specificity of *V. u. rakosiensis*, which is quite stagnant but starts with lower values, and the sensitivity of *N. natrix*, which remains around 90% even at 25% of vertebra completeness. The specificity of *C. austriaca* remains near 100% at every level of vertebra completeness, and its sensitivity declines stronger than that of the other species.

3.3. Identification of vertebrae from nests

We found $n = 14$ snake vertebrae in 5 out of the sampled *Buteo buteo* nests ($n = 15$). We found no snake vertebrae derived from the sampled *Circus pygargus* nests ($n = 3$). We found $n = 191$ snake vertebrae in all the sampled *Circaetus gallicus* nests ($n = 4$).

Based on the simulation results, we discarded the identification results of unknown vertebrae with vertebra completeness lower than 50% in the case of presacral and lower than 80% in the case of caudal vertebrae. These specific thresholds were selected because, at these levels, all species consistently demonstrated a specificity of 90% or higher.

With this approach, we identified $n = 10$ of the total vertebrae belonging to *Coronella austriaca*, $n = 172$ of the vertebrae as *Natrix natrix* and $n = 23$ of the vertebrae as *Vipera ursinii rakosiensis* (Fig. 5). The vertebrae samples collected from *B. buteo* nests were 85.7% *N. natrix* and 14.3% *V. u. rakosiensis*. The vertebrae samples collected from *C. gallicus* nests and pellets were 5.2% *C. austriaca*, 83.8% *N. natrix* and 11% *V. u. rakosiensis*.

4. Discussion

Our approach of identifying snake vertebrae based on the geometric morphometric framework successfully classified samples during the simulation, therefore can be used as a method to identify unknown samples. We proved that the collection of nest and pellet material to study raptor bird diet for finding rare prey like snakes is a feasible method. We confirmed that *Buteo buteo* and *Circaetus gallicus* prey on *Vipera ursinii rakosiensis*. However, in the case of *Circus pygargus* we did not find any snake remains in the studied samples despite of former direct observation of the predation of *V. u. rakosiensis*. It is important to

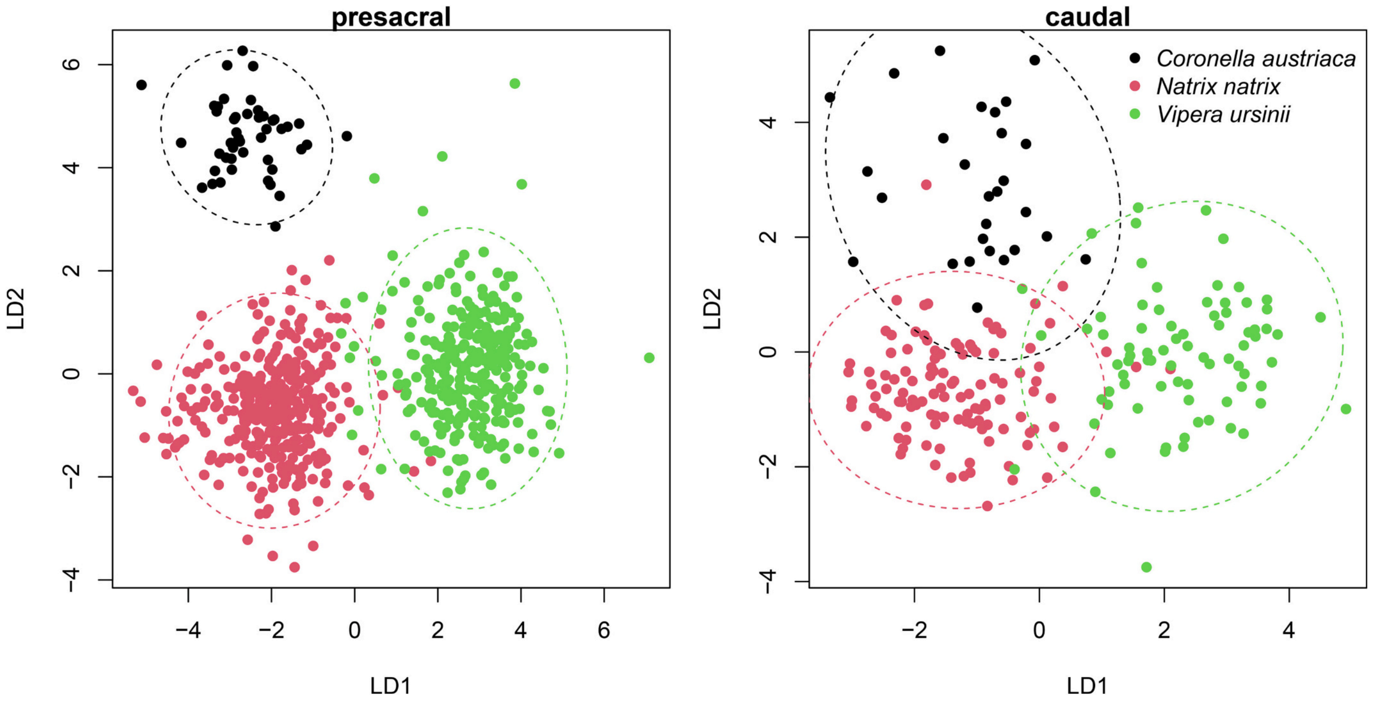

Fig. 3. Visualization of linear discriminant analysis on reference vertebrae.

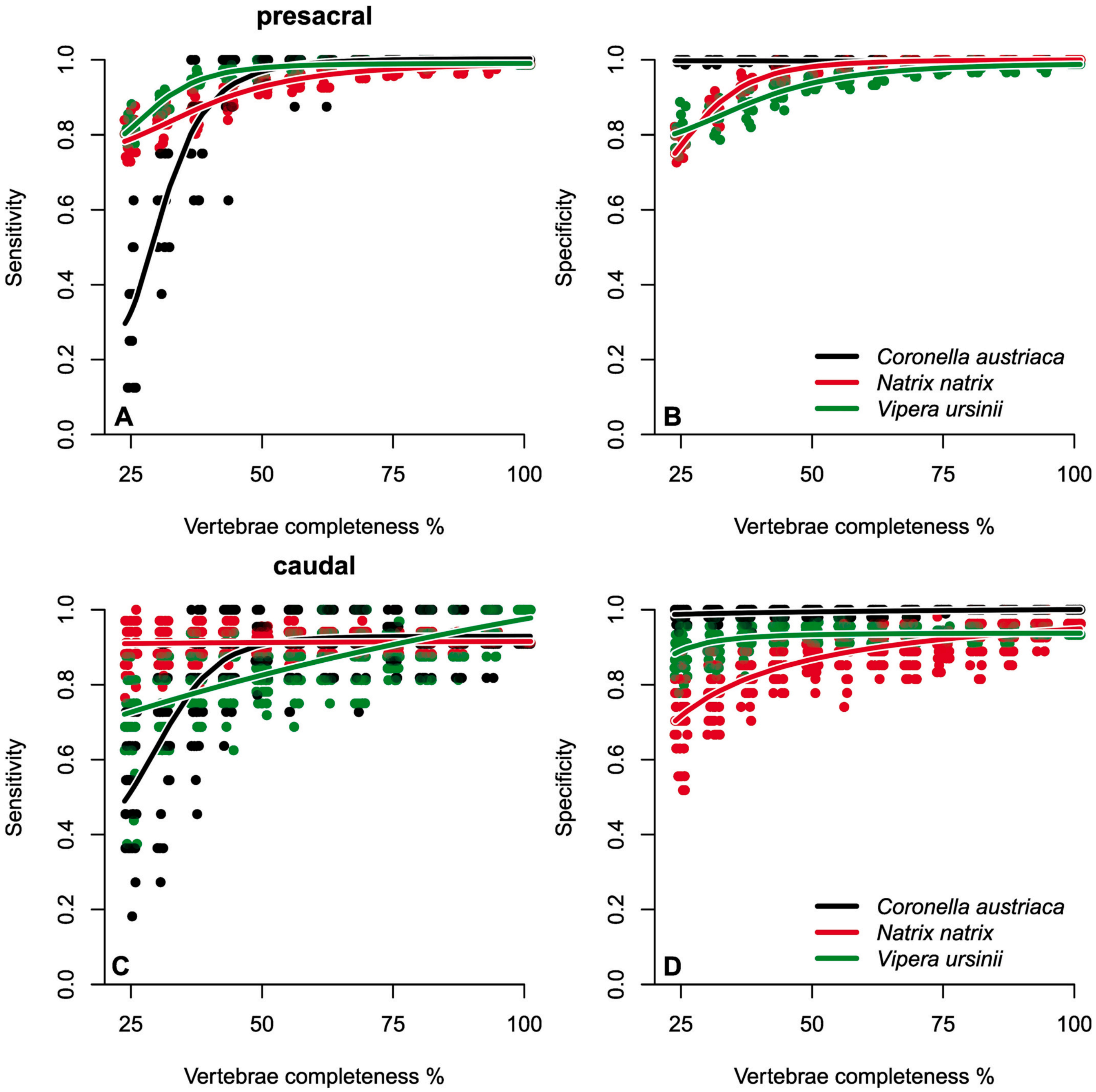

Fig. 4. Efficiency testing of identification with simulations based on vertebra completeness. Presacral vertebrae: A, Sensitivity of identification; B, Specificity of identification. Caudal vertebrae: C, Sensitivity of identification; D, Specificity of identification.

note, that only one of the three nests was in close vicinity of *V. u. rakosiensis* habitats and *C. pygargus* has a smaller hunting range than *C. gallicus.* While all *C. gallicus* samples contained snake vertebrae, in the case of *B. buteo* only a third of them did, which is in agreement with the former's reptile specialist, and the latter's generalist diet (Haraszthy, 2022; Papp et al., 2022). We found that *Natrix natrix* remains were the most abundant, which is ecologically plausible, especially since it is the primary prey of *C. gallicus* in Hungary (Haraszthy, 2022).

Species identification was accurate (90% or higher) when vertebrae had a completeness of at least 50% in the case of presacral ones and at least 80% in the case of caudal ones. This classification rate is remotely high compared to other studies using geometric morphometry (Ibañez et al., 2007; Mutanen and Pretorius, 2007; Sauer et al., 2020). Even though vertebrae are sturdy, they are often damaged in various parts due to digestion or deterioration, therefore the ability to objectively identify them, even when they are incomplete, is particularly useful. Nonetheless, our results indicate that certain vertebrae cannot be unambiguously identified, especially the ones with low vertebra completeness. Caudal vertebrae show a larger overlap between species and are more variable in their intraspecific morphology, whereas presacral vertebrae are generally easier to identify.

Our results support the supposition that local avian predators maintain high predation pressure on *V. u. rakosiensis*, even though we did not measure it systematically. Most of them are generalists and thus can exploit the snakes' thermoregulatory needs and potentially destabilise viper populations that already struggle with low abundance, while

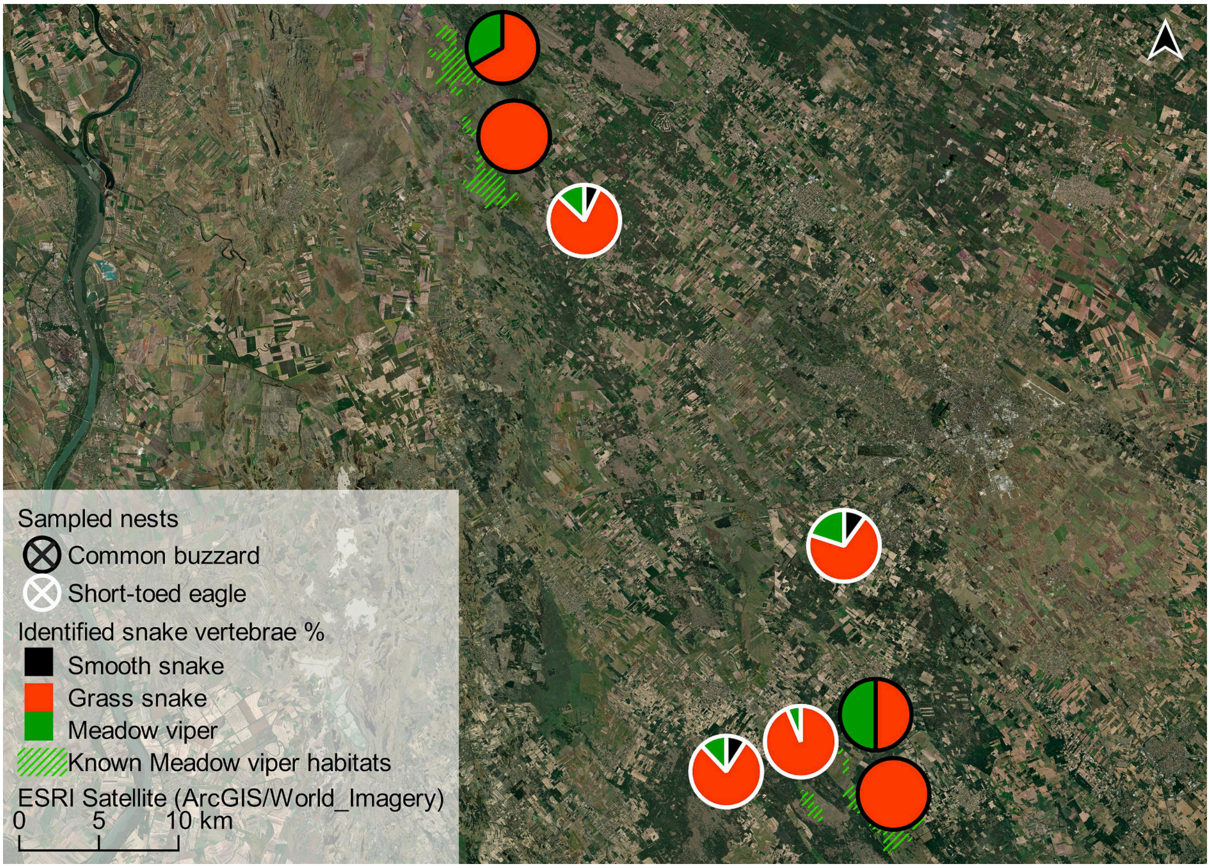

Fig. 5. Identification results of collected snake vertebrae.

refraining from significantly decreasing prey availability for themselves. Possible conservation actions to stabilize viper populations are habitat development (e.g., removing perches, restoring original vertical vegetation structure), predator management on game species, and predator exclusion.

This study provides a well-functioning method to identify the species of snake prey based on vertebrae obtained from predatory feeding remains. To measure predation pressure on given snake species, nests, burrows, latrines, and other areas used for feeding or defecating by potential predators could be located and sampled periodically, in a non-invasive way, therefore they can provide with continuous feeding remains as samples during the active periods of the studied snake species. After processing the samples and identifying the potentially acquired vertebrae with the described method, the results will show the number of vertebrae of each species found per sampling period at all sampling locations. However, since the spine of a snake usually can consist of more than a hundred vertebrae, the number of vertebrae does not accurately reflect on the number of specimens caught by the predator. On the other hand, the prevalence of recovering given species' vertebrae can provide invaluable data about the quantity of which predator at what locations hunts it successfully and can be compared with the estimated size of local populations. It might also show which exact periods snakes are more prone to become prey, therefore specify sensitive periods of species of interest. In addition, frequency data such as this can be efficiently analysed with basic statistical tests as well as complex multivariate models.

Even though analysing the lateral side as well as the ventral one or pairing geometric morphometry with traditional morphometry might further enhance the accuracy of identification, our goal was to find the most efficient and practical way of snake vertebrae classification used for applied conservation. Using geometric morphometrics on just one side of a vertebra proved to be sufficiently accurate, therefore further forms of analysis can be omitted. Regarding *C. austriaca,* we could only study one specimen's vertebral column as a reference, therefore we possibly did not cover the whole morphological variability of the species.

5. Conclusion

In conclusion, by developing and applying this method we successfully identified intact and incomplete snake vertebrae from raptor bird nests, which in the long term, with an increased number of sampled nests, will tell us more about the temporal dynamics of predator-prey interaction among raptor birds and snakes such as *V. u. rakosiensis* to maximize conservation efforts. The landmark selection, the identification process, the efficiency testing simulation, and the described sampling method is all implementable to other studies and snake taxa as well, therefore this method has the potential to be a dependable tool for species identification of snakes in any ecological context where high predation pressure poses a threat and vertebrae can be recovered.

CRediT authorship contribution statement

Ádám Tisza: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Conceptualization. **Attila Móré:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Zoltán Turny:** Funding acquisition, Investigation, Resources, Validation. **Attila Bereczky:** Investigation, Resources, Validation. **Zoltán Szentesi:** Conceptualization, Methodology, Resources, Validation. **Zoltán Korsós:** Conceptualization, Supervision, Validation. **Edvárd Mizsei:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Data availability

The original data of this research and the code used can be found at https://doi.org/10.5281/zenodo.8270331.

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