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CONFERENCE PAPER

The use of strontium isotopes ($^{87}Sr/^{86}Sr$) to determine the geographic assignment based on keratin values of shed skins of green pythons (*Morelia viridis*) as an effective tool against wildlife crime

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Abstract
The green tree python is quite a favorite pet for sale on the international market. The species is therefore protected by the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). Since the illegal poaching of large numbers of specimens in the wild might lead to the detriment of native populations, and wildlife breeding farms were found to be serving as conduits to funnel wild-caught green tree pythons out of Indonesia, a forensic tool to distinguish wild-caught from captive-bred specimens could support the enforcement of CITES protections. To disrupt the illegal trade of green tree pythons, we have developed an effective tool to distinguish the animals supposedly bred in captivity from those caught in the wild, based on the strontium isotope composition in conjunction with trace element data. Like in human hair, $^{87}Sr/^{86}Sr$ values seem to vary according to the relative contribution of endogenous and exogenous sources. Thus, we infer that if there is enough sustainable strontium available for the analysis, it might be possible to use the $^{87}Sr/^{86}Sr$ values in parallel with trace elements to distinguish wild-originated specimens from the in captivity-bred ones. Indeed, our pilot study on the shed skins of animals where the geographic origin was either the Czech Republic or Indonesia, confirms that shed skins can be effectively used for further forensic Sr radiogenic isotope analyses.

Introduction

Many animal and plant species are traded internationally nowadays and therefore the risk of overexploiting their populations by international trade has been regulated by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1976 (CITES Trade Database 2023; UNEP 2023). This trade includes an exchange of live animals, often traded as pets. Reptiles, including snakes, belong to the most often traded favorites (Janssen 2021). Green tree pythons (*Morelia viridis*; Schlegel 1872) native to New Guinea, adjacent islands of Indonesia, and the Cape

York Peninsula of Australia, are among very often traded live reptiles both internationally and locally (CITES Trade Database 2023; Kusrini et al. 2021; Mutiaradita et al. 2023; Toomes et al. 2022). Although there is an intra-species variability related to the geographic origin, the exact attribution of the individual color morphs to the individual geographic location might be questionable (Maxwell 2005; Natusch et al. 2020), though among breeders the color morphs are usually marked with the locality names, e.g. Biak, Aru, Jaya, Kofiau, Arafak, etc. This attribution of the green tree python's color morph to the geographic place might be tested by modern methods of provenance, such as radiogenic isotope analysis. However, the disputable effect of the locality on the color morph will be lost when the animals are bred in captivity, when only the possible genetic effect of the color morph will remain, but the effect of the local food chain will be unified.

Individual signatories of the CITES might set country-specific rules for specific species, which is the case of the green tree python. Since 1979, Indonesia has banned the trade of wild-caught green tree pythons, although the trade of captive-bred individuals remains legal (Maxwell 2005). Australia has banned the export of live specimens since 2000 (Government of Australia 2021). Recorded legal Indonesian export of live green tree pythons, the biggest world export of this snake, covers 119,000 specimens between 1977 and 2022 (CITES Trade Database 2023). Former investigations brought evidence of the laundering of wild specimens through the breeding farms (Lyons and Natusch 2011). This practice seriously threatens the wild populations (Gore et al. 2019), but there are not many tools that would help to distinguish the true origin of the specimens (Moré et al. 2014; Pernetta 2012). Since stable isotope analyses (e.g. N, C, O, S isotopes) have successfully been used to track animal migrations and reveal the geographic origin (DeNiro and Epstein 1978; Hobson and Wassenaar 2008), it appears that it might provide the reliable forensic evidence helping to distinguish wild from captive-bred animals as well (Alexander et al. 2019; Brandis et al. 2018; Natusch et al. 2017; van Schingen et al. 2016; Ziegler et al. 2018). Radiogenic strontium isotope ratio ($^{87}Sr/^{86}Sr$) analyses is already used in forensics, especially to identify the origin of human remains, food, or drugs (Coelho et al., 2017; Kelly et al. 2005; Kramer 2022) or in ecology to identify animal migrations (Reich et al. 2021). Strontium in human hair keeps the same levels throughout the longitudinal growth of the hair strain in individuals that do not migrate (Rodiouchkina et al. 2022) showing that there is no physiological discrimination of the Sr isotopes. However, unlike all animal tissues, the radiogenic $^{87}Sr/^{86}Sr$ values show spatial distribution, as Franke et al. (2008) found for beef and chicken meat. Additionally, heavy and many transition metals values (e.g. vanadium, manganese, cobalt, nickel, copper, zinc, arsenic) were found to be higher in skins of wild-originated Burmese pythons in Indonesia when compared to captive-bred ones, but this did not apply in the reticulated pythons in Viet Nam (Natusch et al. 2017).

So far, no study on the distribution of radiogenic strontium isotopes has been done on shed skins of reptiles, so therefore we performed a pilot study to unravel its potential usability in reptile-related forensic studies. In parallel, we also determined barium, rubidium, and lead concentrations in shed skins of green tree pythons to conclude whether they might serve as suitable material for the concentrations or isotope studies and whether the detected levels of strontium and other elements might discriminate geographically different animals. Barium, rubidium and lead contents in green tree pythons are lacking but can provide additional valuable information about the life and environment of green tree pythons. For example, barium (Karraker and Welsh 2006) is an important element in the food chain reflecting the diet composition of the snakes and possibly environmental contamination. In comparison, rubidium (Karasov and Diamond 1988) that behaves similarly to K in nature could provide information on the snakes' metabolism and their ability to regulate electrolytes. Finally, since lead (Sparling et al. 2010) is a toxic element that could have a negative impact on snake health, its presence could indicate environmental lead contamination in areas where snakes are found. Therefore, combining Sr, Ba, Rb and Pb concentration data in snake shed skin could provide a more comprehensive view of the living conditions, diet and health status of snakes in different locations. This information could be also useful for the conservation and management of snake populations.

Our final aim is to develop a forensic method for distinguishing captive-bred from wild-taken animals. The big challenge of the analysis performed on live specimens is devising a harmless and noninvasive sampling method, which seems to be easier in reptiles, where repeated skin shedding occurs. Shed skins preserve environmental information from the reptile's recent past within their keratinous structure (Kufnerová 2021). However, to reconstruct a longer-term life history, other body tissues may need to be examined (Weber et al. 2002).

Materials and methods

Samples and solution analysis ICP-MS

In our pilot study, we analyzed 14 shed skins of 9 individual green tree pythons (further referred to as Chondro 1 to Chondro 9) that were part of a private collection of shed skins available to Charles University. Three specimens were housed in the Czech Republic for more than 2 years prior to the analysis (Chondro 1, Chondro 2, Chondro 3). Three shed skins were collected from Chondro 1 and Chondro 2; six animals were imported from Indonesia to the Czech Republic (Chondro 4 to Chondro 9), and their first shed skins after the import were collected in the Czech Republic, and out of these in one case the second consecutive shed skin was collected (Chondro 5) as well.

The samples were cut out of the whole shed skins with scissors, cleaned with a 2:1 chloroform-methanol mixture, washed and dried for 24 hr at 40°C. Scissors were each time washed with ethanol between individual samples to overcome sample cross-contamination.

At the Laboratories of the Geological Institutes of the Faculty of Science, Charles University, the cleaned shed skins weighing 200 mg were dissolved in a 25 mL PP flask using 3 mL 14 M HNO_3 and 1 mL 30 % H_2O_2 with the addition of Milli-Q water. Afterwards, the elemental analyses of barium, rubidium, strontium and lead, were performed using the ICP-MS iCap-Q (Thermo Fisher Scientific) following the protocol detailed in Strnad et al. (2005). The calibration curves were prepared with blank and multi-element stock reference standard solutions (Analytika Ltd., Czech Republic).

Sr isotopic analyses

The Sr isolation and mass spectrometry analyses were carried out at the Institute of Geology of the Czech Academy of Sciences (IG CAS). To achieve the lowest blanks, the purest grades of acids available (double Teflon distilled HNO_3 and HCl, H_2O_2 ultrapure grade Sigma Aldrich, and suprapure grade H_3PO_4 Merck) were used.

First, about 15 mL of stock solution used for trace element analyses was transferred to 30 mL Teflon beakers and dried. The residue was then redissolved two times in a mixture of 14 M HNO_3 and 31% H_2O_2 (5 mL total), dried again and finally dissolved using 1 mL 1 M HNO_3. The Sr separation from the matrix was performed using an ion exchange chromatography following the methods detailed in Pin et al. (2014) using a double-cleaning procedure of Sr resin to maintain the lowest blanks. Strontium was collected using 2 mL of 0.05 M HNO_3 after passing elemental matrix through the columns using multiple rinsing steps of 1 M and 7 M HNO_3. The Sr isotopic compositions ($^{87}Sr/^{86}Sr$) were determined by a TIMS technique using the Triton Plus instrument (Thermo Scientific) housed at the IG CAS. The samples were dissolved in 1 μL of 1 M HNO_3 and loaded onto W filaments in the presence of a TaF_5 activator (Charlier et al. 2006). The collection of Sr ions was performed in a static mode using Faraday detectors connected to 10^{11} Ω amplifiers while mass fractionation was corrected using the $^{88}Sr/^{86}Sr$ ratio = 8.375209. The external reproducibility of Sr isotopic analyses was monitored by periodic measurements of the NIST SRM 987 yielding an $^{87}Sr/^{86}Sr$ ratio of 0.710243 ± 0.000014 (2σ, n = 31).

Results

The analyzed $^{87}Sr/^{86}Sr$ ratios show (Figure 1) no significant difference between the group of snakes housed in the Czech Republic for a long time (CZ) and those imported from Indonesia (ID), but the

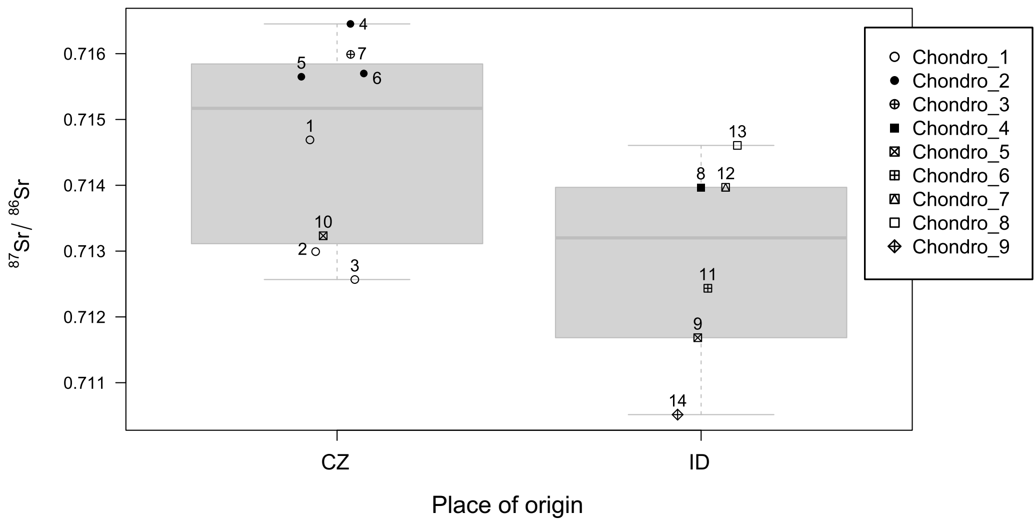

Figure 1. *The boxplot of $^{87/86}$Sr ratios for the individuals divided according to the declared origin of the shed skins. ID – Indonesia, CZ – Czech Republic.*

tested groups did not represent an ideal statistical dataset (small number of animals, repeated and non-repeated shed skins mixed, background information unknown).

Reliable values of strontium contents were detected in all studied samples (minimum 1.52 mg/kg, maximum 13.4 mg/kg) while their ^{87}Sr/^{86}Sr ratios vary from 0.710515 to 0.716452. Both findings are in line with results used in forensics reported elsewhere (Coelho et al. 2017). Other elements (Figure 2) in shed skins that have been measured at higher concentrations are Rb (10.5–50 mg/kg), Ba (1.5–57 mg/kg) and Pb (0.3–3.0 mg/kg).

Discussion

Assumptions and usability of strontium isotopes to track down the movement of snakes

Snakes living in an area with geological background that is characterized by high Rb/Sr ratios and therefore, radiogenic ^{87}Sr/^{86}Sr values, would mimic this signatures in their bodies (Coelho et al., 2017; Kelly et al. 2005; Kramer et al. 2022). If the isotopic composition of the snakes' skins matches the isotopic composition of the underlying bedrock, it can be inferred that the snakes lived in this area. In turn, if the isotopic composition of the snakes' skins does not match the bedrock isotopic composition, it can be concluded that the snakes lived in a different area. Therefore, this method can be used to track the movement of snakes through time and space considering that the composition of the skins of the snakes living in breeding farms will be unified throughout the breeding facility (provided that the same snake species, though in different color morphs, will be on the same diet, which is an effective management measure). On the other hand, snakes living in the wild, in different locations, might show different isotopic compositions, or a wider range of values, as Natusch et al. (2017) have shown for Burmese pythons.

The use of radiogenic ^{87}Sr/^{86}Sr as a forensic tool is of particular interest because unlike stable isotopic systematics of the light elements (e.g. O, N, H), biological mass-dependent fractionation the ^{87}Sr/^{86}Sr directly reflects the Sr isotopic composition of the source. In addition, their wide amplitude of variation on both large and small scales, low temporal variability, and detected relatively high abundance of Sr make ^{87}Sr/^{86}Sr a strong candidate for tracing inorganic and organic materials, either independently or in conjunction with tradional stable isotopic data.

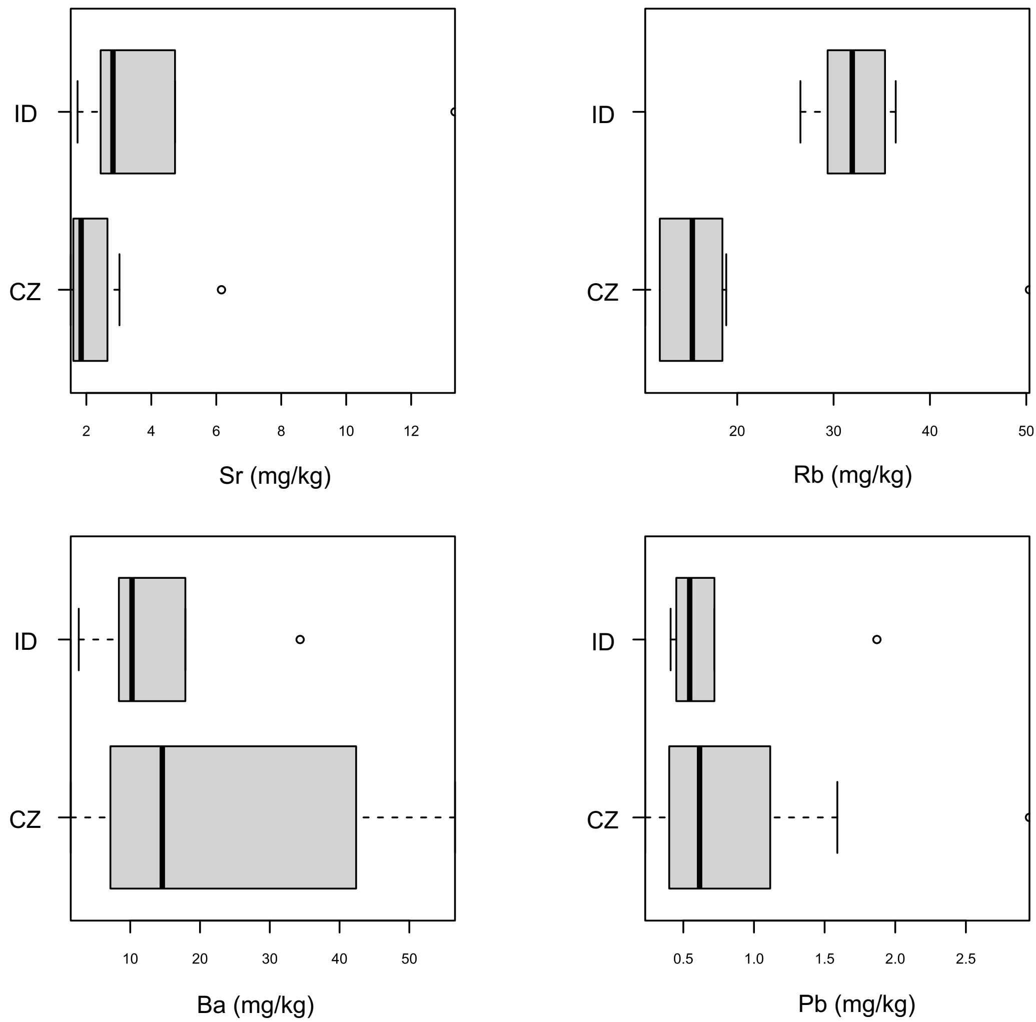

Figure 2. *The boxplots of concentrations of Sr, Rb, Ba, and Pb respectively, of the shed skins, divided according to declared origin. ID – Indonesia, CZ – Czech Republic.*

In human hair, $^{87}Sr/^{86}Sr$ values seem to vary according to the relative contribution of endogenous (diet) and exogenous (ambient humidity, dust, and other contaminants) sources (Hu et al. 2020). It has been inferred from these findings that the $^{87}Sr/^{86}Sr$ values of wild skin might deviate from in captivity-bred ones if contaminants of dietary sources add a distinct strontium isotope signal to keratin. Given the paucity of data for strontium isotopes in wildlife species in general and the green tree python in particular, it is important to test for a possible fractionation effect and the possibility of mixing different Sr sources of strontium isotopes in the shed skin of the green tree python.

The number of individuals in our study is too low for proper statistical analysis, however, the pilot data show that the $^{87}Sr/^{86}Sr$ ratios might tend to differentiate between localities, though these differences are not significant. Additionaly, no information is available about the breeding conditions, the effect of feeding, or geological background of both localities and therefore, further and more detailed studies with all these information included are needed to explore all these possible effects on determined Sr isotopic compositions.

It might appear that the limitation of the presented method is the size of the snake: baby snake's shed skins do not reach the tested 200 mg weight. Shed skins of smaller-sized baby snakes might weigh as low as 100 mg, as found in our collection of shed skins. However, this limitation can be easily overcome by using a TIMS protocols developed for a very low Sr sample weights that were recently tested and adopted (e.g. Scheiner et al. 2022).

The ecological limitation of the strontium analysis of the shed skins for forensic purposes lies in the fact that the snake needs to be usually kept for a period of several weeks or months in controlled conditions until the snake sheds its skin. This might be seen as a disadvantage by the enforcement bodies, however, it is much more in line with the ethical approach and welfare of the animals, rather than sampling invasively by cutting the tip of the tail.

The exchange and mixing of Sr between the skin (Sr from food) and the environment (Sr from rain, mud, soil, etc.) is a complex topic that requires more in-depth investigation. Rodiouchkina et al. (2022) has shown that Sr exchange occurs in keratin, and this issue should be better addressed in this study. Given this fact, it is possible that the skin has a Sr value reflecting the bioavailable strontium in the area (i.e., the animal's diet) and that of rainwater (typically around 0.7092).

Therefore, a Sr value in the skin that differs from that in the local area does not necessarily mean that the animal is not native to the area. On the other hand, a $^{87}Sr/^{86}Sr$ value that is outside the range of the local area and stormwater (the term used for precipitation water that falls on the Earth's surface during rain or snowmelt and this water does not soak into the ground but runs off the surface into drains, rivers, lakes or other bodies of water) would indeed indicate that the animal's skin gathered its Sr isotopic signature outside of the site of discovery.

It is important to consider the various factors that can influence Sr values in animal skin. For example, animals that migrate between areas with different Sr signatures may exhibit values that do not correspond to the local environment. In addition, the Sr isotopic composition in the skin may be influenced by physiological processes such as metabolism and excretion (Flockhart et al. 2015).

The geological Sr elemental and isotopic composition is different from the biologically available signal that is received through the diet. This phenomenon is essential for the correct interpretation of strontium isotope data obtained for stripped shed python skins. It is important to note that the geological signal represents the total strontium signal derived from the rocks and overlying soils in a given area, but not all of this strontium is bioavailable to plants and subsequently to the animals that consume these plants. Various factors such as the solubility of strontium-bearing minerals (e.g. feldspar, apatite), soil pH and the selectivity of plants for nutrient uptake affect the amount of strontium that eventually enters the food chain (Burger and Lichtscheidl 2019).

It is therefore necessary to establish appropriate baseline values for a given area that reflect the bioavailable strontium signal. These values should be based on an analysis of the current fauna and flora of the region, or on the analysis of archaeological remains of animals known to have been present in a limited area.

Without these baseline values, interpretation of data obtained from human remains may be misleading. For example, if the geological signal in an area differs from the biologically available signal, this could lead to erroneous conclusions regarding the migration and mobility of individuals in the past. The background information on the sites (isotopic composition of the underlying bedrock, water, precipitation, and prey), both on the breeding facilities and on the place of wild origin, will complete the picture and serve for better construction of a high quality forensic tool.

Conclusion

Strontium isotopic compositions ($^{87}Sr/^{86}Sr$) can be used to track the movements of snakes over time and across different regions. This method is based on the assumption that snake shed skins should have the same isotopic composition as the geological background of the area where they live. If the isotopic composition of the snakes' shed skin does not match the isotopic composition of the underlying geology, then it can be concluded that the snakes had lived in a different area. For forensic purposes, it may be sufficient to disprove the origin claimed by a suspected illegal trader, without needing to establish the exact true origin in court for successful enforcement.

Chondro 5 was the only individual imported from ID, where 2 consecutive sheddings were recorded. It seems that the results obtained from the very first shed skin (4 weeks after the import) were closer to the "ID group," but the second shed skin, when the individual was already living in Czechia for a period of 6 weeks, was closer to the "CZ" group. Our results suggest that the change in Sr ratio can be rather fast, and it corresponds with the finding of the Rodiouchkina's work that Sr can be replaced. For the possible forensic use then only the first shed skin seems to be important, however, the small group of data in this pilot study does not allow for precise assessment.

Radiogenic strontium isotope composition ($^{87}Sr/^{86}Sr$) is a particularly useful tracer for this purpose since it is not affected by biological or instrumental mass-dependent fractionations. Additionally, $^{87}Sr/^{86}Sr$ values have a wide range of variation in nature, which make them useful for tracking both large and small-scale mobility.

The shed skins can be used for sampling and subsequent radiogenic and stable isotopes analysis since they contain enough strontium, barium, lead, and rubidium contents needed for the analyses. However, the size matters—the researcher needs to control the number and character of analysis that will be performed. In addition, a reserve sample still needs to be stored for further unexpected analysis, which is a good practice in all forensic analyses.

The shed skins on their own do not provide complete information, and additional background information needs to be collected for correct data interpretation. Analysis of other elements might serve as good supportive evidence, but the basic regularities of their distribution and turnover need to be verified in practice. Finally, our study suggests that the values of strontium, rubidium, barium, and lead detected in shed skins can potentially serve also in further forensic studies, although a larger number of specimens of known origin should be analyzed and reported to set reliable forensic tools.

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Competing Interests. The authors declare no conflicts of interest.

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