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Lungless salamanders of the genus *Speleomantes* in the Solling, Germany: genetic identification, *Bd/Bsal*-screening, and introduction hypothesis

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Abstract. We report on an introduced population of Italian Cave Salamander (*Speleomantes italicus*) that has been living in an abandoned quarry of the Solling area, Germany, within a beech forest near the town of Holzminden, since at least 2013. DNA sequences of the mitochondrial genes for 16S rRNA and cytochrome *b* confirm these specimens being genetically assignable to *S. italicus*, without genetic differentiation from populations of the native range. Nine individuals studied for infection by chytrid fungi by quantitative PCR tested all negative, both for *Bd* and *Bsal*. Up to 18 specimens per night were seen, and small juveniles (35 mm total length) observed from 2015–2020, suggesting continued successful reproduction. To understand the origin of this introduced *Speleomantes* population, it is relevant to consider the existence of an animal trade business in Holzminden until 1977 that imported, among many other amphibians and reptiles, *Speleomantes italicus* as early as 1914; furthermore, the same family business also exploited up to three quarries from 1946 to 1956 in the same area, although we could not verify whether this includes the one currently populated by *Speleomantes*. Although no proof exists that this business is related to the introduction, it is plausible to hypothesise that the introduced cave salamanders have persisted at the site for decades, maybe even for more than a century, given that the quarry populated by these salamanders exists since at least 1896 based on historical maps. The population appears to be highly localised and not invasive, and at present does not appear to represent any danger for native animals or plants. Eradication therefore does not appear to be necessary, and we emphasise that according to German law also introduced populations of wild animals benefit from protection, unless lifted as for instance in the management of invasive species.

Keywords. *Speleomantes italicus*, allochthonous population, Plethodontidae, introduced species, chytridiomycosis

Introduction

Plethodontidae represents the largest family of salamanders including more than 490 species inhabiting predominantly the American continent, including North, Central and northern South America (Amphibiaweb, 2021). In addition, eight cave salamander species of the genus *Speleomantes,* closely related to the North American *Hydromantes,* are native to Europe (Speybroek et al., 2020); and one species, *Karsenia koreana*, is known from South Korea (Min et al., 2005). The European plethodontids occur in France and Italy where they inhabit the Maritime Alps, the central Apennine Mountains in continental Italy, and Sardinia. Since all plethodontids lack lungs, respiration takes place solely through their moist skin. Several species have a ballistic tongue which they can project up to 80% of their body length (Deban et al., 2007). Besides, they feature a vertical slit between the nostril and upper lip known as the "nasolabial groove" which is lined with glands. The specific structure of the groove enhances the salamander´s chemoreception and enables a maximised drainage from the nose to counterbalance the lacking lungs (Brown and Martof, 1966).

European cave salamanders require high moisture and relatively cool temperatures (Ficetola et al., 2012). Therefore, these are mainly nocturnal and subtroglophile species with a poor dispersal ability inhabiting caves, stone quarries and artificial cavities, where they can find their preferred environmental conditions throughout the whole year in the Mediterranean region (Lanza et al., 2005). They are active between 2.6 °C and 22 °C, and at

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a relative humidity between 67 and 100% (Lanza et al., 2005). Mating and reproduction occur on land, without an aquatic larval stage. The female lays a clutch of eggs in a crevice out of which fully developed salamanders hatch. Lunghi et al. (2014) reported the first observation of brooding and maternal care in *S. italicus* and *S. flavus* in the wild. They highlighted the preference for deep sectors of caves that feature small cavities in vertical walls to reduce the risk of predation as well as the need of stable microclimate conditions for successful egg development that requires nearly one year. Due to their cryptic way of life, knowledge about the behaviour and life history of *Speleomantes* is scarce, and human-mediated introductions of these animals may therefore remain unperceived over considerable amounts of time. Introduced *Speleomantes* populations are known from several locations in Italy and France (Lucente et al., 2016), and most recently, *S. strinatii* has been discovered in west-central France in the Vienne county, more than five hundred kilometres outside its known distribution range, apparently the result of human introduction (Lucente et al., 2016).

Here, we report the discovery of European cave salamanders at an abandoned sandstone quarry in the Solling area in Lower Saxony, Germany (Fig. 1), which they have been inhabiting for at least seven years (2013–2020).

Discovery, habitat and life history of *Speleomantes* in the Solling, Germany

The Solling constitutes a range of hills up to 528 m above sea level. It belongs to the Weser Uplands and is located in the state of Lower Saxony, with its southerly foothills extending into the States of Hesse and North Rhine-Westphalia. After the Harz Mountains, it is the second largest massif in Lower Saxony, being covered mostly by spruce and beech forests. Large parts of the Solling are protected as the Solling-Vogler Nature Park.

The first discovery of *Speleomantes* in the Solling was made by Julius Zidorn in 2013, and reported on the website fieldherping.eu. The photographed specimen was confirmed to belong to *Speleomantes*, and preliminarily identified as Italian Cave Salamander, *S. italicus,* in the forum of this website. The population was subsequently visited by numerous hobbyists and field herpetologists. The site is located close to the small town of Holzminden; for conservation reasons, we here do not provide exact geographical coordinates, but only state that the site is located less than three km from the city centre (coordinates 51.83°, 9.46°).

Upon a visit of our team on 3 May 2015, an estimated 10 specimens were observed in the quarry (Fig. 1C,D), during constant and heavy rains active at night, both within crevices in the quarry rock wall, and on the ground, on tree roots and on rocks. These included several very small specimens, which we hypothesise were juveniles of 1–2 years in age at most.

During a night with no rain but distinctive humidity on 3 June 2015, around 10 *Speleomantes* were observed of which at least one was a juvenile (Fig. 1G). The sighting of the first salamander took place at approximately 22:00 h, i.e., at dusk just about half an hour after sunset. Most of the salamanders sat in crevices in the rock walls (Fig. 1E) only their heads being visible while others were walking on ledges of the stone wall. We found one salamander a few meters away from the rock wall being situated on the forest ground (Fig. 1F). This individual was recorded at about 2:30 h which indicates that it had enough time in that night to move from the rock wall towards the site of observation. This animal also stood out because of its rather brownish pattern, without reddish elements as in most other *Speleomantes* individuals observed at the site.

Various other visits to the site also consistently yielded observations of *Speleomantes*, as long as a certain air and soil humidity was present. Weak and constant rain and temperatures of 10-17 °C appeared to be the ideal conditions to observe larger numbers of individuals (maximum number was 18 individuals observed on one night). The salamanders were typically observed at night in total darkness, starting ca. 1 hour after sunset, but exceptionally, single individuals could sometimes already be seen a bit earlier. Juveniles and sub adults were regularly seen, and both sexes were recorded in approximately similar proportions. No individuals could be observed upon visits in December 2016 at cold and dry weather (air temperature 3-4 °C), and in summer 2019 after a long dry period, but at least two adult specimens were found on the stone wall outside of the crevices during rather dry weather on 25 August 2018 at around 23:00 h (Fig. 1H).

On 4 September 2020, we again observed 11 specimens at the same site during a nocturnal search (Fig. 1B), ranging from 35 mm to 105 mm in total length. While all of the adults were found on the ledges of the quarry, the smallest individual (35 mm) could be observed on the ground in front of the rock wall. The occurrence of small-sized specimens both in 2015 and 2020 suggests a continued reproduction of this population.

According to our observations, the salamanders' presence is limited to one abandoned main sandstone quarry (Fig. 1I) and at least one smaller similar rock structure nearby, both located in rather dense beech forest. Examining topographic maps ("Messtischblatt" 1:25,000 no. 4122, available through the Landesamt für Geoinformation und Landesvermessung Niedersachsen; https://www.geobasisdaten.niedersachsen.de/) revealed that the quarry already existed in 1896 (maps produced as part of the "Preußische Landesaufnahme") in more or less the current extension, and was also mapped in the respective maps from 1937 and 1956. A larger nearby quarry also existed already in 1896, but apparently increased in surface between 1937 and 1956, suggesting active extraction of rocks. Hence, from the available maps there is no evidence for recent mining and extraction activity in the small quarry populated by *Speleomantes* between 1896 until present, and it cannot be excluded that it may have been largely abandoned for more than a century. In the 1896 map, a small area around the quarry is already surrounded by forest but the overall area appeared to be dominated by open pastures, while in 1937 the area was more heavily forested, and increasingly surrounded by settlements.

Apparently, the surrounding forest does not offer suitable conditions for the salamanders to survive since no specimens have been reported outside of the mentioned place. It can be presumed that deep crevices inside of the quarry ensure the animals' survival. Rubbish, graffiti and various kinds of remains of human activities do not seem to hinder the salamanders from thriving in the stone walls.

Genetic assignment of the Solling *Speleomantes* population

Although the specimens in the Solling have from the beginning been assigned to the Italian Cave Salamander, *Speleomantes italicus,* based on their typical colour pattern, they have never been genetically analysed so far. To assess their genetics and better understand their possible origin, we extracted DNA from a buccal swab of one individual using standard salt extraction, and amplified fragments of two mitochondrial genes that previously had been used in *Speleomantes* phylogeographic studies (e.g., Cimmaruta et al., 2015; Lucente et al., 2016): a fragment of cytochrome *b* (cob) was amplified using primers Cytb-a (CCATGAGGACAAATATCATTYTGRGG) and Cytb-c (CTACTGGTTGTCCTCCGATTCATGT) of Bossuyt and Milinkovitch (2000), and a fragment of the gene for 16S rRNA was amplified using primers 16Sar-L (CGCCTGTTTATCAAAAACAT) and 16Sar-H (CCGGTCTGAACTCAGATCACGT) of Palumbi et al. (1991), following standard PCR protocols. PCR products were purified using ExoSAPIT (USB) and sequenced on automated DNA sequencers at LGC Genomics (Berlin, Germany). The newly determined DNA sequences were quality-checked with the software CodonCode Aligner (CodonCode Corporation), and submitted to GenBank (accession numbers MW557805 and MW548269).

For phylogenetic placement of the samples we relied on the cob fragment because a higher number of sequences of this gene were available for comparison. All cob samples of *Speleomantes* from GenBank (accessed in October 2020) were downloaded, aligned to the new sequences, and the best-fitting substitution model (TN93+G) determined based on the Bayesian Information Criterion in MEGA7 (Kumar et al., 2016). We used this model for unpartitioned phylogenetic analysis of the cob sequences under Maximum Likelihood (ML) in MEGA7, with SPR-3 branch swapping, assessing node support with 2000 nonparametric ML bootstrap pseudoreplicates.

Exploratory analyses using the full cytochrome *b* alignment unambiguously showed that the German *Speleomantes* sample was embedded within *Speleomantes italicus,* in agreement with its previous morphological identification. We then trimmed our alignment to a length of 371 nucleotides, which were available from all nine comparative *S. italicus* sequences and from our specimen. The resulting ML tree (see Fig. 2 for the *S. ambrosii/italicus/strinatii* clade) suggests a high mitochondrial uniformity of *S. italicus* across the sampled range in Italy, and the only weakly divergent haplotype co-occurred with a common haplotype in the area of Lucca, suggesting poor phylogeographic structure of this species. The newly sequenced specimen from the Solling had a haplotype identical to the most common one found also in Lucca, Firenze, Ancona, Perugia, Pescara and Reggio Emilia. This makes it difficult to trace more in detail the origin of the introduced German population, but the data confirm unambiguously its identification as *S. italicus.*

Bd-/Bsal-Screening

Numerous species of salamanders, among which *Speleomantes*, are known to be susceptible to infection by either or both of the pathogenic amphibian fungi *Batrachochytrium dendrobatides* (*Bd*) and

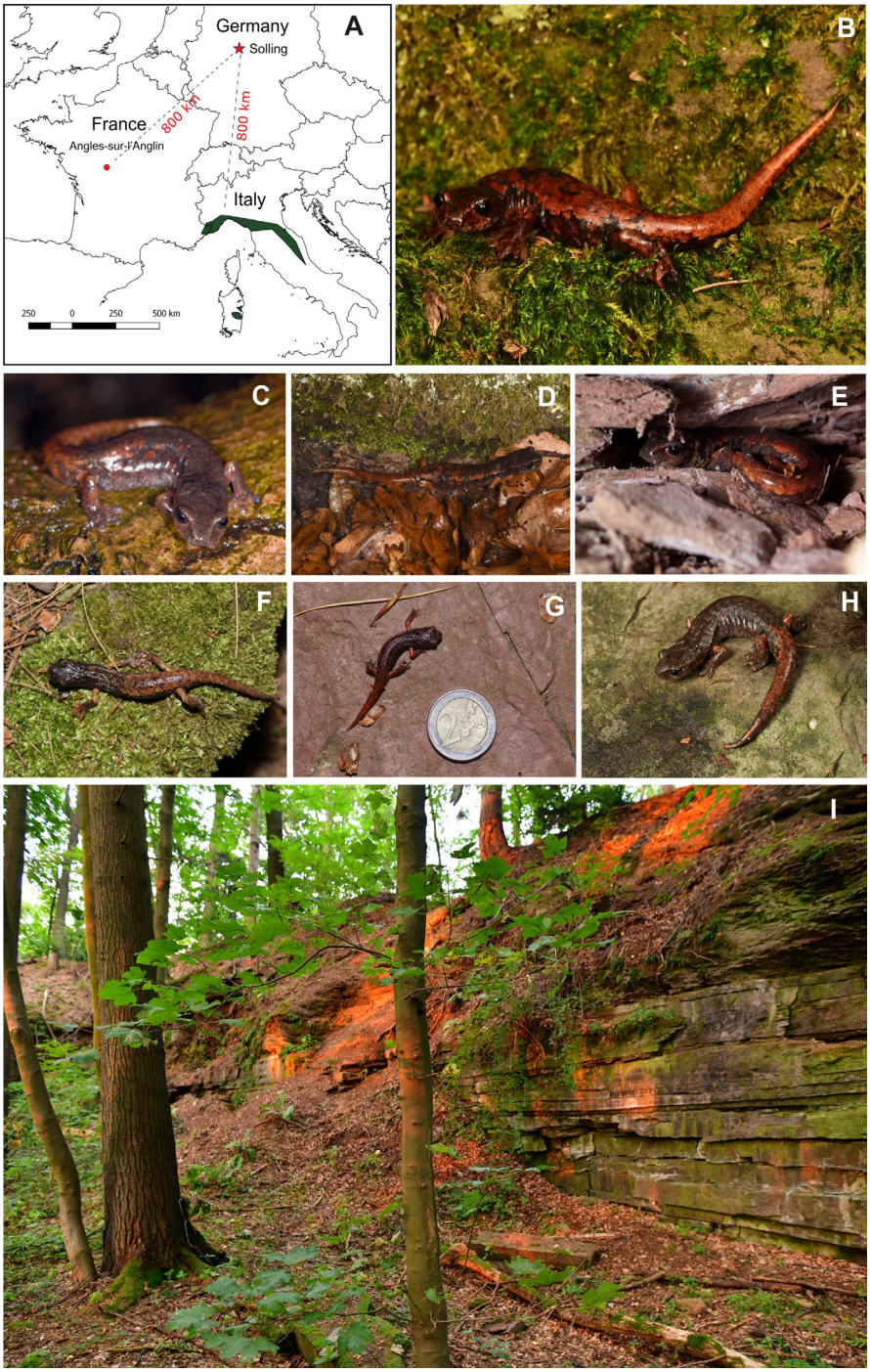

Figure 1. *Speleomantes italicus* from the Solling, Germany. (A) Distribution map showing the new locality of the introduced population, and its distance from the nearest introduced and native *Speleomantes* populations. (B) Individual of *S. italicus* from the Solling, found on 4 September 2020. (C, D) Individuals found on 3 May 2015. (E–G) Individuals found on 3 June 2015, including (E) an individual in the original position in a crevice, (F) a brownish individual found several meters away from the rock wall, and (G) a juvenile. (H) Individual found on 25 August 2018. (I) View (in 2020) of the abandoned sandstone quarry in the Solling where the introduced *S. italicus* population was discovered.

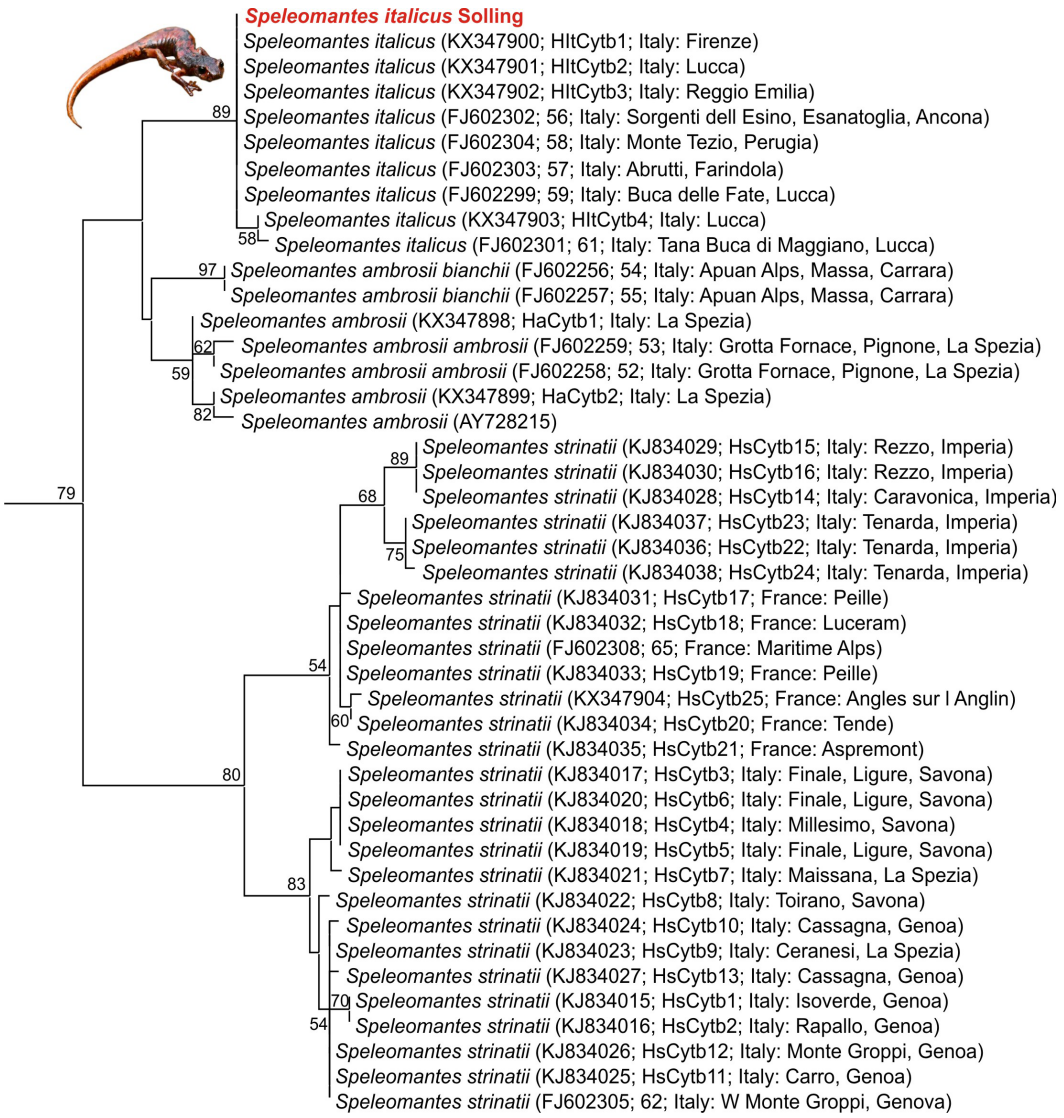

Figure 2. Maximum Likelihood tree based on 371 nucleotides of the mitochondrial cytochrome *b* gene, indicating the placement of the introduced *Speleomantes* sample from the Solling, Germany, with other *S. italicus* from their native range. The tree shows the subclade containing the three related species *S. ambrosii, S. italicus,* and *S. strinatii* and was rooted with the remaining *Speleomantes* species (not shown to improve illustration of relationships within this focal subclade). Numbers at nodes are bootstrap values in percent from an analysis with 2000 replicates.

Batrachochytrium salamandrivorans (*Bsal*) (Vazquez et al., 2009; Martel et al., 2014; Carter et al., 2020). *Bd* affects all clades of amphibians, representing one of the major drivers of global decline in amphibian species (Scheele et al., 2019), while *Bsal* seems to be only lethal to urodelan species (Martel et al., 2014). Although a hypervirulent *Bd* lineage (*Bd*GPL) occurs in the habitat of *Speleomantes* in France and Italy (Bovero et al., 2008; Farrer et al., 2011; Tessa et al., 2013), no *Bd*-infection or *Bd*-related mortality in these species have been reported (Chiari et al., 2013; Pasmans et al., 2013). Surprisingly, Pasmans et al. (2013) revealed that all eight European *Speleomantes* species are resistant to chytridiomycosis, caused by *Bd*, probably due to highly effective fungicidal properties in their skin secretion preventing skin invasion. In contrast, at least one species

of European cave salamander, *S. strinatii*, was lethally affected by *Bsal* in infection trials (Martel et al., 2014), a finding that might indicate a similar susceptibility of the related *S. italicus.* However, so far *Bsal* has not been detected in any wild amphibian population in Italy (Grasselli et al., 2019). To test whether the introduced Solling population of *S. italicus* may be infected by chytrid fungi and thus potentially acting as a vector of these pathogens, we took nine skin swabs for a screening of the chytrid fungi *Bd* and *Bsal* via qPCR following standardised protocols (Blooi et al., 2013). All nine tested individuals revealed negative results for *Bd* and *Bsal*.

Hypothesis on the origin of the introduction of *Speleomantes* into the Solling

How did Italian Cave Salamanders end up in a remote quarry in Germany, and for how long has this population been thriving there? The unambiguous observations demonstrate that this population has been in the area at least since 2013, and very likely is successfully reproducing. As discussed above, the quarry exists since at least 1896, and may have been abandoned since those early times. To trace the source of this introduction, it might be relevant that the Solling has been long known for particular colour morphs of fire salamanders (*Salamandra salamandra*) and therefore attracted the interest of herpetologists in Germany. Already Freytag (1955a,b) reports fire salamanders with aberrant markings like red, high yellow or very dark to entire black specimens from the Solling area. Freytag (1955b) stated that he obtained these striking salamanders from "W. Koch", which probably referred to a family-owned animal trade business located in Holzminden.

As reported in two articles published in "Anzeiger Holzminden" in 1937 and 1938, this family business sold local as well as exotic reptiles, amphibians and other animals, from a place (Rumohrtalstraße 10/12/14, Holzminden) located less than 2 km away from the *Speleomantes* population. Animals were imported and exported across Europe to private keepers or for scientific purposes. The historic German hobbyist journal "Blätter für Aquarien- und Terrarienkunde" includes several classified advertisements posted by the Koch business, offering a variety of reptile and amphibian species. Next to native German and non-European caudates (e.g. *Taricha torosa* from North America and *Cynops pyrrhogaster* from Japan), "*Spelerpes fuscus*" was offered in an advertisement in an edition of this journal from as early as 1914, this name constituting a synonym of *Speleomantes italicus* (see e.g. Gorman, 1956). Another advertisement from 1925 in the journal Wochenschrift für Aquarien- und Terrarienkunde 22 (29) also offers "Höhlenmolche (ital.)", almost certainly referring to *Speleomantes italicus.*

According to information from the Holzminden town archive, the family company was apparently founded before World War I by an elder brother of Fritz (1905–1946) and Walter (1907–1954) Koch (possibly Ludwig; 1895–1918). The company was registered on January 1st, 1932 by Walter and Fritz Koch. After Fritz's death in World War II, the business was continued by Walter Koch, and after his death on 18 June 1954, by his widow Elisabeth and son Alfred. The business was deregistered on 15 October 1977. It is uncertain to which member of the Koch family Freytag and Susebach (1948) and others referred as "L. Koch" since the founder, Ludwig, died already in World War I. However, the business was continued under the name of the founder as is obvious from some advertisements, and therefore, Freytag may have referred generically to the Koch family; in fact, in Freytag (1955b) he mentions W. Koch, likely referring to Walter Koch. Much less likely is the hypothesis that some of these mentions refer to Karl Ludwig Koch, a traveller and writer interested in herpetology that has been in contact with German herpetologists Wilhelm Klingelhöffer and Robert Mertens but to our knowledge was not linked to the Solling area.

Freytag (1978) also published an article about the problems of introducing non-native caudates into nature, listing several incidents but not mentioning a release of *Speleomantes*. We could not find any evidence from the literature or from personal conversations that indeed European Cave Salamanders were released by Koch in the Solling. However, given the special habitat requirements of *Speleomantes*, it is probable that the founders of this population were released by someone with basic knowledge of their biology, which certainly would be the case for an amphibian and reptile trader.

One other important piece of evidence comes from the fact that the Koch family was also involved in the exploitation of quarries. According to the Holzminden city archives, Walter Koch registered for quarry operations ("Steinbruchbetrieb") on 1 May 1946; after his death in 1954 the company remained registered for two years in the name of Elisabeth Koch, and was deregistered on 31 October 1956. The company leased three quarries, of which only one was exploited at a time, by three employees. The quarries were located (1) in the "Altendorfer Feldmark (Börngeberg)", (2) "Auf

der Horst (von Kumlehn gepachtet)", and (3) "Abtlg. 45 Forstamt 1, Holzminden". We could not precisely locate the first one; we assume "Börngeberg" refers to either a hill or to the owner of the quarry, but we could not identify such a site or family name. However, "Altendorf" was the former name of the eastern part of Holzminden as obvious from historical maps, and the "Altendorfer Feldmark" may well correspond to the area where the *Speleomantes* habitat is located. The second site very likely is a former quarry located in a forested area, less than 3 km from the *Speleomantes* habitat. The third site, apparently within forest managed by the Holzminden forestry office ("Forstamt"), could not be located as the numbering of forest sections have changed since 1946. It thus is obvious that the Koch family was very familiar with the quarries in the general area of eastern Holzminden, and may well have at some point exploited a quarry in immediate vicinity to the current *Speleomantes* habitat.

Certainly, the Solling *Speleomantes* population has existed for at least seven years and does still thrive. However, it is intriguing to speculate, given the historical hints, that it may have been present in the area already for decades, potentially for over 100 years if taking into account that the Koch family was selling these animals regularly since 1914 and their habitat existed at least since 1896.

Amphibian introductions and translocations in Europe

Within the last century, human-induced translocation or introductions of *Speleomantes* species within and outside their range for various reasons have taken place (Cimmaruta et al., 1999; Forti et al., 2005; Lanza et al., 2005). Lucente et al. (2016) concluded that introductions may easily result in locally viable populations, provided that the site has subterranean suitable retreats. The new record from Germany, about 800 km north of the known *Speleomantes* range in Italy and France, impressively confirms this statement. It also exemplifies the difficulties in predicting the environmental niches of salamanders – and other organisms – that rely heavily on subterraneous retreats. In these retreats, microclimatic conditions are much more stable than on the surface, allowing salamanders – and their pathogens, such as *Bsal* (Li et al., 2020) – to survive under otherwise adverse external conditions of drought and cold.

Although human-induced introductions of non-native species might not necessarily mean harm for the native ecosystem (Schlaepfer et al., 2011), in general, they confer a substantial potential to wreak havoc on the native fauna. For amphibians, one important point to keep in mind in this context is that emerging infectious diseases are a major driver of the global biodiversity loss (Daszak et al., 2000). This is particularly true in amphibians (Stuart et al., 2004; Martel et al., 2014; Scheele et al., 2019) where the introduction of *Bsal* to Europe is causing severe population declines as well as local extirpations of fire salamanders (Spitzen-van der Sluijs et al., 2013; Schulz et al., 2020; Lötters et al., 2020). Salamander translocations confer the risk of translocation of this fatal disease, as in the emergence of *Bsal* at a Spanish site that was likely triggered by introduction of infected Anatolian newts (Martel et al., 2020). However, it also should be kept in mind that it can become a veritable dilemma to manage allochthonous, or even invasive species, that are threatened (or may become threatened in the future) in their native range (Renet et al., 2020). Pragmatic approaches have been suggested (e.g., Marris, 2014; Heise, 2018), especially for urban environments, which may become sanctuaries for species threatened in their original habitat. Within these new habitats, introductions can even been interpreted as beneficial, leading to higher species richness.

From a legal perspective, if a species is strictly protected in Europe (e.g. for *S. italicus*: FFH Directive Annex IV) but introduced to non-native areas in Germany, the protection status of the introduced populations does not remain the same as in their natural distribution range; for instance, their presence does not extend to protection of their habitat (see example of *Podarcis muralis* in Lower Saxony: Blanke and Lorenz, 2019). Allochthonous populations such as the Solling *Speleomantes* thus are not strictly protected according to German law (§44 Bundesnaturschutzgesetz) but it is important to emphasise that they still benefit from general protection as other wild living animals (§ 39 Abs. 1 Bundesnaturschutzgesetz, §1 Tierschutzgesetz), unless this protection is specifically lifted e.g. in the context of managing or eradicating invasive species. Furthermore, in order to keep such animals that are protected at the European level in captivity, documents proving their legal origin are necessary. Therefore, collecting specimens from such non-native amphibian population for private purposes is illegal.

Partly along with climate change, we are increasingly seeing range extensions of numerous species, which in densely inhabited areas of Europe may take place through a complex mixture of natural and human-mediated dispersal. In this context, disentangling the

origins of particular populations of neozoans will become increasingly difficult, and discussions about their protection vs. eradication will become common. For the *Speleomantes* population in the Solling we emphasise that there is no need to take particular management or even eradication actions, and recommend conserving these salamanders. Importantly, this introduced population involuntarily represents a fascinating field experiment to test long-term survival of a salamander in a macrohabitat bioclimatically drastically differing from those in its natural range. It thus offers an excellent opportunity to study niche conservatism vs. niche shifts at the micro and macro level (Ficetola et al., 2018) in European cave salamanders.

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