RESEARCH ARTICLE

An amplicon sequencing protocol for attacker identification from DNA traces left on artificial prey

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Abstract

1. Clay model studies are a popular tool to identify predator–prey interactions that are challenging to observe directly in the field. But despite its wide use, the method's applicability is limited by its low taxonomic resolution. Attack marks on clay models are usually identified visually, which only allows classification into higher taxonomic levels of predators. Thus, the method is often biased, lacks proof and, above all, standardization.

2. Here, we tested whether precise identification of attackers can be provided by amplification and sequencing of mitochondrial DNA left in bite marks on clay models. We validated our approach in a controlled laboratory study as well as in a field experiment using clay models of a common European amphibian, the European fire salamander *Salamandra salamandra*. DNA-based taxonomic assignments were additionally compared to visual assessments of bite marks.

3. We show that trace DNA of attackers can be routinely isolated and sequenced from bite marks, providing accurate species-level classification. In contrast, visual identification alone yielded a high number of unassigned predator taxa. We also highlight the sensitivity of the method and show likely sources of contamination as well as probable cases of secondary and indirect predation.

4. Our standardized approach for species-level attacker identification opens up new possibilities far beyond the standard use of clay models to date, including food web studies at unprecedented detail, invasive species monitoring as well as biodiversity inventories.

KEYWORDS

clay models, environmental DNA, high throughput sequencing, predator–prey interactions

1 | INTRODUCTION

Predator–prey interactions in ecological contexts can inform about mechanisms of natural selection and ultimately the function and evolution of prey traits. Interaction networks of food webs characterize biological communities, and quantifying their properties is key to the assessment of biodiversity dynamics (Schneider, Brose, Rall, & Guill, 2016). However, predator–prey interactions are difficult to access, because predation events are often cryptic, unpredictable and field observations of predation are usually very rare. Over the last

FIGURE 1 Visual abstract: artificial prey models are placed in the field and attack marks are used for predator identification. Using the common visual assessment of attacks allows (if at all) identification of a predator group. Genetic assessment, as presented in our protocol, allows identification down to the species level

decades, the use of artificial prey models placed in the field has become a widely used tool, mostly but not exclusively, to gain insights into predator–prey interactions (Bateman, Fleming, & Wolfe, 2017; Rößler, Lötters, & Pröhl, 2018).

Models of prey, usually made from non-toxic plasticine clay, are deployed in the field, and attack marks left on them can be ascribed to a broadly taxonomically defined predator type. In addition to identifying the attacker (Willink, García-Rodríguez, Bolaños, & Pröhl, 2014), clay model studies can be used to assess local predation pressure (Roslin et al., 2017) or to test the effect of visual signals such as colours and patterns on predation frequency (Dreher, Cummings, & Pröhl, 2015; Hegna, Saporito, Gerow, & Donnelly, 2011). Clay model studies have been used across a wide range of prey taxa including amphibians, reptiles, mammals as well as various arthropods (Kikuchi & Pfennig, 2010; Leone, Loss, Rocha, Paes, & Costa, 2019; Mason, Wardell-Johnson, Luxton, & Bateman, 2018; Paluh, Hantak, & Saporito, 2014).

Despite their utility, clay model studies have clear limitations (Bateman et al., 2017; Rößler et al., 2018). They rely on a subjective identification of bite marks from visual assessment, which limits predator allocation to broad taxonomic groups and consequently results in a low resolution of the method. The visual identification of attackers is often difficult and likely biased by observer interpretation. Moreover, the method is not standardized in terms of identification criteria, and a high proportion of potential attacks on clay models that remain unidentified usually must be excluded from further analyses despite containing valid information. That said, clay models are also regularly attacked by animals out of curiosity (e.g. due to a novel smell etc.), but not always necessarily because they are truly mistaken as their natural prey. Thus, while we use the term predator throughout this protocol, we urge caution concerning its ecological validity in each given context.

Here we propose a solution to this issue by applying eDNA analysis to clay model studies. Recent developments in high throughput sequencing technology have revolutionized biodiversity research. Whole biological communities can now be routinely characterized by metabarcoding (Thomsen & Sigsgaard, 2019; Yu et al., 2012) and species interactions can be tracked at unprecedented detail (Kennedy, Lim, Clavel, Krehenwinkel, & Gillespie, 2019). Species can also be identified indirectly from eDNA (Ficetola, Manenti, & Taberlet, 2019; Rees, Maddison, Middleditch, Patmore, & Gough, 2014; Taberlet, Bonin, Zinger, & Coissac, 2018; Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012). All organisms leave traces of DNA in their environment, for example in faeces or saliva—of

which even minuscule amounts can be detected by species-specific quantitative PCR or by amplicon sequencing (Bohmann et al., 2014; Pompanon et al., 2012). Bite marks constitute a promising source of eDNA, as has been previously shown in forensic analyses (Sundqvist, Ellegren, & Vilà, 2008; Sweet & Shutler, 1999) as well as in ecological studies (Monge, Dumas, & Baus, 2020). Recently, salivary DNA of brown bears was isolated from salmon carcasses (Wheat, Allen, Miller, Wilmers, & Levi, 2016) and DNA analysis of bite marks on livestock helps to distinguish wolf and domestic dog attacks (Sundqvist et al., 2008). Thus, we also expect to find residual attacker DNA left on imprints in clay models.

Here, we test whether eDNA of predators can be routinely detected from bite marks left in clay models and subsequently used to identify the attacker. For this purpose, we performed two experiments, based on targeted PCR amplification and amplicon sequencing of attacker DNA. First, we tested the general possibility of routinely isolating predator DNA in a controlled test targeting dog bite marks in clay balls. Second, a clay model study on the European fire salamander (Amphibia: Caudata: *Salamandra salamandra*) was conducted under field conditions. The European fire salamander is a common, predominantly nocturnal species that is mainly predated on by various mammals, such as red fox and wild boar (Seidel & Gerhardt, 2017). Clay models with presumed mammalian as well as unidentifiable bite marks were collected. We then taxonomically identified attacks on the models, by extracting, amplifying and sequencing DNA from bite marks. Specifically, our aim was to compare the efficacy of visual taxonomic assignments of bite marks based on characteristic imprints and DNA-based assignments (Figure 1).

2 | MATERIALS AND METHODS

2.1 | Standardized test of eDNA recovery from bite marks

In order to assess the general applicability of our method, we performed a controlled test with a domestic dog in the home of one of the authors (SL). Prior to the experiment, we confirmed with the responsible state veterinarian that our procedure does not constitute a case of animal testing and does not require further permits. There was also a cat present in the home, which we used to assess the effect of random environmental contamination. We prepared 10 equally sized clay lumps from black, non-toxic plasticine

modelling clay (Noris Club®; Staedtler) using sterile gloves. The dog was encouraged to voluntarily bite each of the lumps, which were then transferred to sterile zip lock bags and transported back to the laboratory. Sterile razor blades were used to slice out small pieces of modelling clay from each bite mark. The clay pieces were transferred to 1.5-ml tubes and DNA was extracted using the Qiagen Puregen Tissue kit (Qiagen) according to the manufacturer's protocols. To account for the expected low yield of DNA, we added Glycogen as a DNA carrier during DNA precipitation and reduced the volume of the final elution to 30 μl. Blank DNA extractions were included to account for contaminations in the modelling clay.

We then used a universal primer pair targeting a short fragment (~100 bp amplicon length, deepening on taxon amplified) of mitochondrial 12S rDNA of mammals (Karlsson & Holmlund, 2007) to amplify dog DNA and possible other contaminants in the DNA extracts. All extracts were amplified in 10 μl reactions, at an annealing temperature of 46°C and at 35 cycles using the Qiagen Multiplex PCR kit, according to the manufacturer's protocols. Amplification success was checked on a 1.5% agarose gel. Following the first round PCR, we added an indexing PCR with five cycles to incorporate 8 bp dual indexes and sequencing adapters to the amplicons. Indexing PCRs were performed following the primer sets described in Lange et al. (2014) using the Qiagen Multiplex PCR kit. After indexing, all samples were checked on a 1.5% agarose gel and then pooled in approximately equimolar amounts based on band intensity on the gel relative to a ladder and to each other. The final pool was cleaned from leftover primer using the High Pure PCR Product Purification Kit (Roche).

2.2 | Clay model production and field experiment

European fire salamander clay models were made from the same black non-toxic plasticine modelling clay as used above with the help of previously designed silicon moulds made from a realistic hard plastic fire salamander cast (Bullyland; #68493). Patterns were hand-painted with non-toxic acrylic ink (AERO COLOR® Professional; Schmincke). All steps of model production and subsequent handling were conducted using gloves to reduce contact contamination with human DNA. A total of 800 models were deployed into the field along transects with a minimal distance of 7 m between models. The experiment was conducted in a broadleaf forest near Trier University in Germany (49°41′29.3″N 6°47′31.2″E), where we verified the presence of *S. salamandra* prior to the study. Models were placed in the forest between 22 May 2018 and 1 July 2018 and were checked for attacks every 96 hr by two observers. Attacked models were replaced with a new model until the end of the study to maximize the number of attacks. The attacked models were collected in sterile plastic bags, photographed on scale paper and visually assessed for bite marks. Where possible, predator types were allocated at the time of collection. Clay models were subsequently frozen at −80°C.

2.3 | Visual assessment of attacks

Visual inspection included assessment by two observers (always the same) and comparison with specimens (toothed skulls) from the educational collection of Trier University. Available skulls included: Cetartiodactyla: wild boar *Sus scrofa*; Carnivora: red fox *Vulpes vulpes*, stone marten *Martes foina*, badger *Meles meles*; Rodentia: ship rat *Rattus rattus*. The assessment was based on visually identifying characteristic traits of teeth and jaws that could explain imprints found on models. Other than comparison with toothed skulls, the assessment potentially included a subjective identification based on experience as well as considering the potential force/body size needed to deform the model. However, visual assessment, as in previous studies, lacked a clear standardization.

2.4 | Confirmation of eDNA recovery from field-collected bite marks of known predators

To confirm the presence of attacker eDNA on field-deposited clay models, we first targeted samples with bite marks that could be clearly assigned to a predator species known to prey on European fire salamanders (Carretero & Rosell, 1999; Seidel & Gerhardt, 2017). Due to their tusks, wild boars *S. scrofa* leave a very distinctive bite mark, unlikely to be confused with other mammals in the study area (Figure 4). We thus chose a subset of 10 clay models with clearly identifiable wild boar bite marks.

Using an alignment of *S. scrofa* sequences downloaded from GenBank we designed a primer pair targeting a 189 bp fragment of the mitochondrial COI gene (Supporting Information Table). As slugs of the genus *Arion* (not amphibian predators) were commonly observed to feed on clay models in the field, we included various *Arion* COI sequences into the alignment. Primers were designed to suppress the amplification of *Arion* spp. based on mismatches at the 3′-end (see Krehenwinkel et al., 2019). The designed primer sequences (5′-3′) were F: TTGTTACAGCTCATGCCTTTGTA and R: GCTTCTACTATTGAGGATGCCAG. DNA was extracted from the models and the DNA extract subsequently amplified using the previously designed *S. scrofa* COI primer pair as described above. PCRs were run with 40 cycles and at annealing temperatures of 46°C. Amplification success was checked on a 1.5% agarose gel and samples that amplified were cleaned up using the High Pure PCR Product Purification Kit (Roche). Cycle sequencing was then performed using the ABI BigDye kit (Applied Biosystems) and the samples were sequenced on an ABI capillary sequencer (Genetic Analyzer 3500; Applied Biosystems). Sequences were edited in Codon Code Aligner (Codon Code Cooperation) and compared to *S. scrofa* COI reference sequences to confirm their identity. All clearly identified *S. scrofa* bite marks yielded interpretable Sanger sequences. Sanger sequencing was chosen, as this method was readily available in our institute.

2.5 | eDNA recovery from field-collected bite marks

After we had confirmed the suitability of our eDNA protocol on known predators (*S. scrofa*) of field-deposited clay models using Sanger sequencing, we used Illumina amplicon sequencing to identify other predator species on clay models with visible mammalian bite marks (Supporting Information Table). We used the previously described mammal-specific 12S rDNA primer (Karlsson & Holmlund, 2007). To check the reproducibility of amplicon sequencing results and test the possibility of multiplex PCR targeting multiple loci, we included another mammal-specific primer pair targeting 16S rDNA (Karlsson & Holmlund, 2007). Both used primer pairs amplify a fragment of ~100 bp, with some size variation between taxa. We included samples from bite marks that were previously taxonomically identified as well as those that were not identifiable but were assessed as constituting an attack. Previous DNA extracts of wild boar attacks were also included in the reaction. One of the samples showed several very small bite marks, most closely matching the ship rat skull. To test the capability of our protocol to reproducibly recover eDNA even from very small bite marks, we performed two independent DNA extractions from bite marks for this model. DNA extraction and PCR amplification (35 cycles), indexing PCR (5 cycles), pooling and purification of the pooled sample were performed as described above. The primers were checked for possible dimers using the Multiple Primer Analyzer tool (Fisher Scientific) and no dimers could be identified. PCRs were then run for some test samples separately for each primer pair and checked on a gel for equal amplification intensity. Multiplex PCR was then run with equal amounts of each primer pair. Negative control PCRs were run along all samples and sequenced as well.

2.6 | Sequencing and sequence analysis

The two pooled rDNA libraries (dog- and field-collected clay models) were sequenced on an Illumina MiSeq (Illumina), each using a Truseq Nano V2 kit with 300 cycles at the Max Planck Institute for Evolutionary Biology in Plön, Germany and according to the manufacturer's protocols. Demultiplexing was done using CASSAVA (Illumina), with no mismatches allowed. Demultiplexed reads were merged using PEAR (Zhang, Kobert, Flouri, & Stamatakis, 2013) with a minimum quality of 20 bp and a minimum overlap of 50 bp during merging. Merged reads were filtered with a quality threshold of >Q30 for >90% of the sequence and transformed to fasta files using the FastX toolkit (Gordon & Hannon, 2010). The two loci (12S and 16S) were then demultiplexed based on primer sequences using *grep* in UNIX. PCR primers were then trimmed off all reads using *sed* in UNIX. After primer trimming, the quality filtered and merged sequences were de-replicated and operational taxonomic units (OTUs) generated using USEARCH (Edgar, 2010) at an OTU radius of 3%. The de novo Chimera removal tool of USEARCH was used to remove chimeric sequences. Taxonomy was assigned to the resulting OTUs using BLASTn (Altschul, Gish, Miller, Myers, & Lipman, 1990) and comparing against the complete NCBI database. BLAST was run with a maximum of 10 target sequences. We removed all non-vertebrate sequences from the dataset, which included bacterial, fungal and nematode reads. OTUs were only retained when they matched a vertebrate with ≥95% similarity. Species ID was only assigned to an OTU, if it matched a reference sequence with ≥98% similarity. Human DNA, which co-amplified for some samples, was also removed, as we could clearly identify it as contamination. OTU tables were then generated for all samples and using the vertebrate OTU sequences as reference. Some taxa yielded more than one OTU matching database sequences for the same species with high similarity. However, one of these OTUs was usually highly overabundant compared to the remaining ones (>10-fold). Also, we found a clear pattern of correlation between the occurrence of the rare OTUs and the main OTU matching the same species (significant correlation of read abundances between rare and abundant OTU samples; linear model in R, Figures S2). Considering that the 12S rRNA and 16S rRNA genes evolve relatively slowly, it is unlikely that these rare OTU sequences constitute true haplotypes. We thus assumed they are NUMTs (pseudogenes of nuclear mitochondrial DNA) (Song, Buhay, Whiting, & Crandall, 2008) and removed them from the further analysis.

To estimate the levels of contamination from handling and environmental contamination under controlled conditions, we used the dog bite data. We compared the proportion of recovered dog, cat and human reads, with the latter two representing possible contaminant sources. Negative control samples were analysed together with all clay model data, as an additional baseline of potential laboratory contamination. The list of recovered taxa was also compared with species commonly studied in our laboratory (particularly bats) to distinguish possible laboratory contamination from true predation events.

3 | RESULTS

3.1 | Standardized test for eDNA recovery from dog bite marks

On average, the number of reads per sample in this experiment was 479 ± 392 ($M \pm SD$, $n = 10$). Dog DNA made up the majority of reads in each sample ($98.83 \pm 1.08\%$ of reads; Figure S1). The most abundant contaminant sequence was human DNA with $0.83 \pm 0.81\%$. In addition, we discovered cat DNA in two samples at low abundance (0.09% and 1.39% of reads). A single sample contained one read of cow DNA (*Bos taurus*) and another one four reads of wild boar *S. scrofa*. The blank extraction and negative control PCR sample contained considerably fewer reads than the actual samples (9 and 29), of which 7 and 8 were classified as dog, 2 and 16 as cat, 0 and 4 as human, and 0 and 1 as wild boar.

3.2 | Mammalian and unidentified bite marks on field samples

Of the 800 deployed clay models, 53 showed clear attack marks and were thus further analysed for the presence of eDNA. We successfully isolated, amplified and sequenced attacker DNA from 45 of these models for the 12S rDNA amplicon. On average, we recovered 5,935 ± 3,161 reads for the 12S dataset per analysed specimen. After removal of NUMTs and human sequences, 4,762 ± 2,741 reads remained per sample. A total of 15 OTUs were recovered across the samples. The OTU sequences were classified into six orders, 11 families and 13 species of mammals. Most of these taxa are common to German forests. All except one OTU sequence could be classified to species level.

For most samples, one taxon was most abundant and dominated the read population. We scored this most abundant taxon as the most likely attacker. Attacker DNA sequences amounted to 97.58 ± 4.32% of the total reads for each sample on average (Figure 2; Supporting Information Table).

For our analysis, the remaining low-abundance reads in each sample were classified as likely non-attacker contaminant sequences. Alternatively, the low-abundance OTUs might derive from the DNA of second attackers on the same model. Only a single sample showed DNA sequences of two almost equally abundant taxa, a European mole *Talpa europaea* (58.45% of reads) and a shrew species *Sorex araneus* (32.78% of reads). In addition to these two species, we also detected DNA of a buzzard *Buteo buteo* (1.76% of reads) in that

sample, suggesting that the 12S primer pair does amplify birds to a certain degree.

The two most frequent attackers were red fox *V. vulpes*, which was classified as attacker for 25 samples and wild boar *S. scrofa*, which was classifieds as attacker for 18 samples. Additional attackers included a shrew *S. araneus*, a badger *M. meles* and a roe deer *Capreolus capreolus*. The sample containing the roe deer DNA also detected DNA of a European hedgehog *Erinaceus europaeus* in low abundance. After reinspection of the attack mark, which recovered deer DNA, it turned out to be a hoof print. The shrew was recovered from the sample, which was visually classified as rat attack and was extracted twice. Both replicate DNA extractions recovered similar results, suggesting that those bite marks were caused by a shrew.

With about 5% of the read population, possible contaminant sequences were more abundant in the field experiment than in our controlled laboratory study using a dog. In some specimens, we found sequences of an unidentified bat species (probably horseshoe bat, *Rhinolophidae*, only 95% database match; 0.02% of reads,), cow (*B. taurus*, 0.45% of reads), sheep (*Ovis aries*, 0.02% of reads), a possible horse (*Equus* sp., 0.005% of reads), domestic dog (*Canis lupus*, 0.40% of reads) and wild boar (0.84% of reads) at low abundances. Most other possible contaminant sequences belonged to red fox (1.89% of reads).

In contrast to the 12S dataset, the 16S data yielded considerably fewer reads for many samples, probably due to differential amplification efficiency in multiplex PCR. We thus selected only a subset
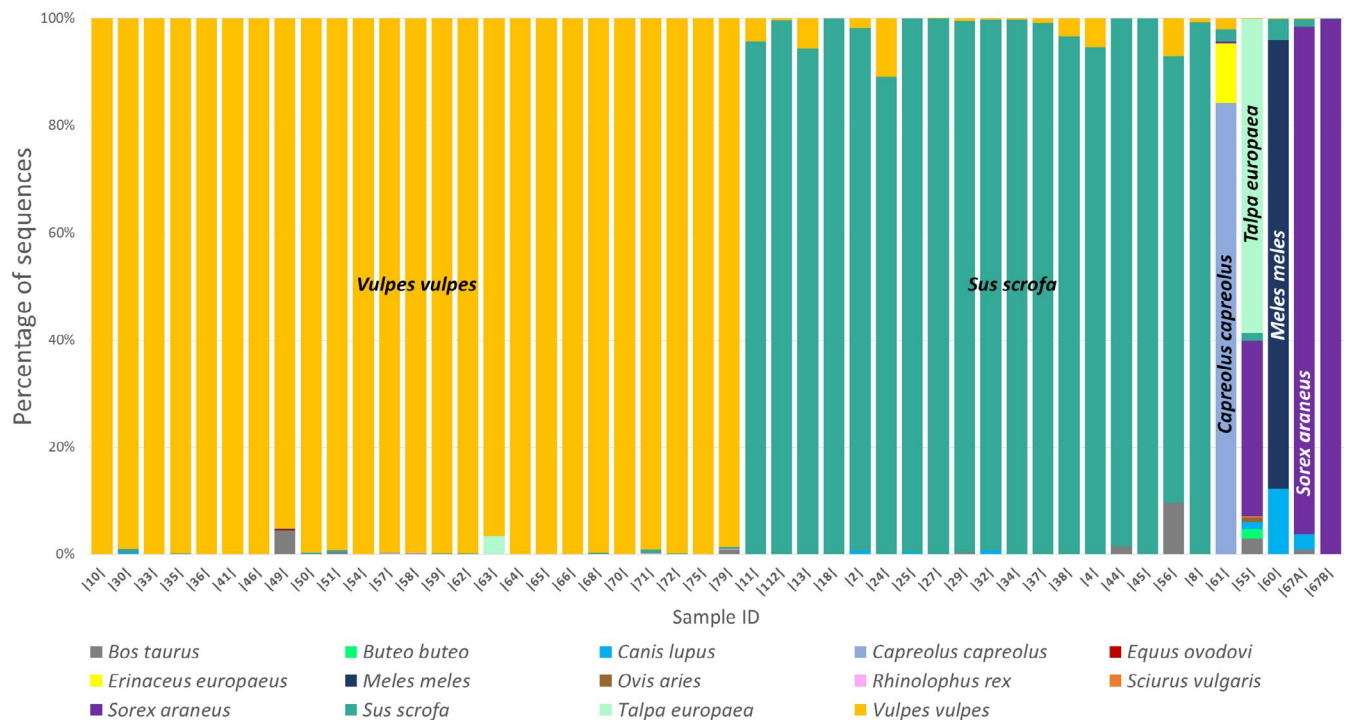
FIGURE 2 Percentage of recovered 12S rDNA reads of different species for all analysed salamander clay models (note that 67A and 67B are replicates). Taxonomy is shown to the lowest possible level and samples are ordered according to the most abundant recovered taxon (see Supporting Information Table)

of 25 samples for further analysis, which had an average coverage of 691 reads per sample. After cleaning, 516 reads per sample remained on average. In total, the 16S data yielded eight mammal OTUs in four orders, eight families and eight species. Most taxa recovered in the 12S dataset were also found for the 16S data. Moreover, all attacker identifications for the 16S exactly matched the 12S data (Figure S3). The average read abundance for attacker sequences was 96.52 ± 4.60%. Even the 12S sample that recovered a European mole and an unidentified shrew species, as well as the DNA of a buzzard, recovered the same species for 16S (*T. europaea*: 15.74%; *S. araneus*: 15.28%; *B. buteo*: 5.56% of reads). However, for the 16S data, that sample also recovered a considerable proportion of DNA sequences of a common frog *Rana temporaria* (62.96% of reads). As for the 12S data, the two replicate extracts of a rat-like attacker turned out as European shrew *S. araneus*. 16S contaminant sequences were mostly made up of wild boar (1.00% of reads) and red fox (1.20% of reads). Cow DNA was only recovered in one sample at very low frequency (0.05% of reads). The *Rhinolophidae* bat was not discovered, but another bat species was found in the 16S data at low frequencies (Serotine bat, *Eptesicus serotinus*: 0.08% of reads on average). This bat species was also dominating the negative control, with 15 out of 22 reads of that sample.

## 3.3 | Comparison of visual and genetic attacker identification

Among all samples used for genetic analysis, 15 could not be allocated to a predator by visual assessment a priori (28%), whereas the remaining 38 samples were identified as either wild boar (34), red fox (2), marten (1) or rat (1) based on attack marks (Figure 3). In 19 samples the genetic result matched the visual assessment, whereas in 14 samples, the genetic assessment of the predator identity was different from the visual assessment. From the 15 samples that could not be identified by visual assessment, we successfully isolated and sequenced attacker DNA in 11 samples. All samples, their visual and genetic assessment can be found in Supporting Information Table.

4 | DISCUSSION

4.1 | Predator identification from eDNA in clay model bite marks—Promises and further optimizations

Clay model studies are widely utilized for a range of ecological and evolutionary questions, with a particular focus on predator–prey interactions (Bateman et al., 2017). As a field-based method, clay models carry many advantages: they can be manipulated to target-specific predators, can include inanimate objects (e.g. eggs, nuts, fruits etc.; Hanson, Newmark, & Stanley, 2007) and could even include olfactory cues in addition to shape and colour. Yet, their main limitation to date is the uncertainty and bias in interpreting bite marks. Our eDNA-clay analysis now addresses this limitation. Genetic identification of predators from imprints provides considerably improved taxonomic assignments.

The recovered attacker species red fox, wild boar and badger are all mammal taxa preying on European fire salamanders in the wild (Seidel & Gerhardt, 2017). Shrews are also known to attack and eat salamanders (Brodie, Nowak, & Harvey, 1979), hence their discovery is not surprising. However, we also discovered some unexpected attacker species. As subterranean hunters, moles do not attack salamanders. Interestingly, we discovered DNA of a common buzzard along with European mole DNA on the same clay model. The mole constitutes a considerable part of the buzzard's diet (Graham, Redpath, & Thirgood, 1995). Hence, the most probable explanation is that a buzzard, which had previously preyed upon a mole had subsequently attacked the clay model. Mole DNA from the bird's beak or talons would then have been transferred onto the model. The abundance of buzzard DNA on the model probably exceeded that of the mole; however, as we used mammal-specific primers for PCR amplification, this likely yielded a strong amplification bias towards the mole. The same reason could apply for the frog DNA detected from that model. *R. temporaria* is a common prey item of buzzards (Martin, 1990). However, this must be interpreted with care, as amphibian DNA is commonly analysed in our laboratory. A roe deer is another unexpected attacker species. But after closer inspection of the 'bite mark' it turned out to be a hoof print. Thus, the source
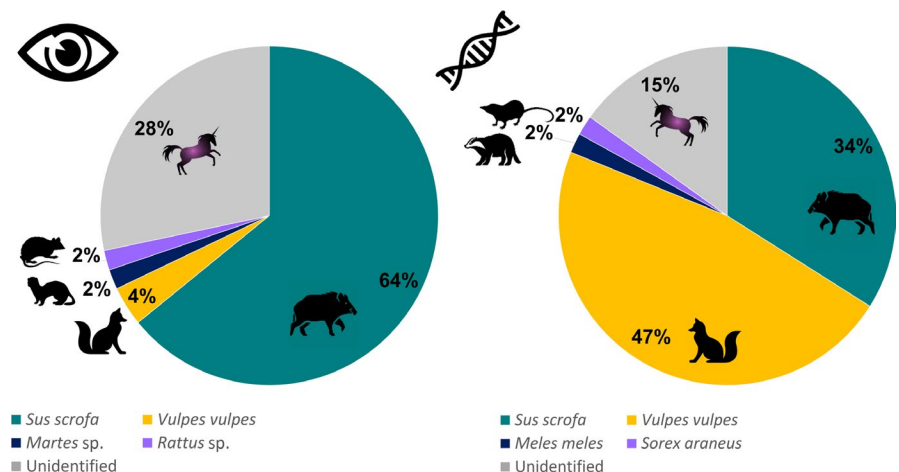
FIGURE 3 Visual (left) and genetic (right) assessment of mammalian attack marks (*n* = 53). The percentage of unidentified attacks decreases from 28% in the visual to 15% in the genetic assessment

of deer DNA must have been a deer stepping onto a clay model. Consequently, due to its sensitivity, the method may also detect secondary predation events and simple contact events not constituting an attack. The genetic data thus need to be carefully interpreted and ideally cross-validated by visual identification of attack marks.

Other unexpected sequences, which we recovered in very low abundance in some samples, belonged to bats. The sequences were most likely laboratory contaminants, as bat DNA is processed in our laboratory on a regular basis. This shows that the eDNA methodology is prone to contamination, even by very small amounts of trace DNA. This is also supported by our controlled experiment using a dog. The mere presence of a cat in the house with a dog was sufficient to leave traces of feline DNA, even in the negative control samples. We also detected likely contaminant DNA traces of foxes and wild boars in many samples, suggesting that some cross contamination may have occurred during field collection or laboratory processing. This may have happened during photographing of specimens, where the same grid paper was repeatedly used for different specimens. Also, the same gloves were used to handle specimens in the field, which constitutes another likely source of cross contamination. When handling clay models in the field, one should avoid touching any bite mark directly or better still use repeatedly cleaned tweezers to handle models. Models need to be handled and transported with as little contact as possible, and should be processed in laboratories, where DNA of probable predators has not been present before.

A further methodological consideration concerns the DNA sampling method. We sliced pieces of modelling clay directly from bite marks and extracted DNA from the slices. Alternatively, swabbing protocols (Mingo, Lötters, & Wagner, 2017; Sweet, Lorente, Lorente, Valenzuela, & Villanueva, 1997) could be used to simplify and standardize the DNA extraction process and avoid damage to the model. Swabbing can be directly performed in the field and additionally avoids the need for further handling of specimens and thus reduces the contamination risk.

Due to the inherent contamination risk, it is also advisable to perform PCR replicates in the analysis (Ficetola et al., 2015; Ficetola, Taberlet, & Coissac, 2016). Only samples that yield attacker sequencing data from multiple amplifications should then be scored as a true attack. Our analysis also suggests that the inclusion of a second primer pair can further improve the method's validity (see also Krehenwinkel et al., 2019; Krehenwinkel, Kennedy, Rueda, Lam, & Gillespie, 2018). Both 12S and 16S supported identical attacker identifications, additionally supporting the recovered results. Using more than one marker also alleviates amplification bias between taxa (Krehenwinkel et al., 2018) and may be more suitable to detect instances of multiple attacks on models. Both markers can be amplified in a multiplex PCR. In our experiments, the 12S rRNA primers probably outcompeted those of the 16S during amplification, so further adjustments to the multiplex will be necessary. It may also be advisable to perform PCR for both markers separately, to provide an additional technical replicate to the analysis.

Another possible optimization would be the use of direct PCR, where slices of clay from bite marks could be directly dropped into the reaction tube. This method is well-established in arthropod DNA barcoding (Wong et al., 2014) and may be worthwhile for clay model studies as well. An advantage over DNA extraction-based protocols would be the avoidance of an additional protocol step, which increases the possibility of contamination.

Although probable contaminant sequences were detectable in our data, it needs to be emphasized that the likely attacker DNA was largely overrepresented in all samples. Attacker identification should thus rely on abundant sequences. However, the question remains whether the protocol also allows identification of instances of multiple attacks on the same model. As we extracted predator DNA directly from supposed bite marks, we mostly discovered single predator species. Yet, the sensitivity of the method should also allow to identify instances of multiple attacks. Ideally, DNA should then be extracted separately from different bite or peck marks. Otherwise, it may be difficult to distinguish true predation events from contaminant sequences. In the sequenced amplicon pools, the attacker's DNA was usually largely overrepresented in relation to possible contaminant DNA. Such a homogeneous amplicon pool does not necessarily have to be sequenced using amplicon sequencing. In laboratories, which do not have access to a high throughput sequencing device, attacker identification can also be performed routinely using Sanger sequencing, as we have shown with wild boar samples. Sanger sequencing will also be an attractive choice, when only small numbers of samples need to be processed. However, a clear advantage of amplicon sequencing lies in its recovery of the complete amplicon pool. This way, the purity of the sequence data and potential contaminants can be identified easily. Also, Sanger sequencing would likely fail to detect instances of multiple attacks, where a mixed amplicon pool is generated.

While our method generally worked reliably, we could not sequence predator DNA from all bite marks. eDNA will persist only for a limited time and its detectability can be affected by the environmental conditions (Thomsen & Willerslev, 2015). For example, a rainfall event could have washed off the majority of DNA from the model. It is also possible that a single PCR does not suffice to detect the minimal DNA traces left, especially in cases of small predators. More sensitive protocols, for example nested PCR (Lan, Ossewaarde, Walboomers, Meijer, & Van Den Brule, 1994) may help to more reliably recover DNA from small bite marks.

4.2 | Genetic versus visual assessments

Visual identification of wild boar and red fox, as the most common attackers, was confirmed by our genetic assessment. However, our genetic results also uncovered a high rate of apparent misidentification from visual analysis and revealed the origin of previously unidentifiable attack marks. Thus, the eDNA-clay protocol can be a highly effective method to complement visual assessment, to standardize the identification process, to prove assumptions and to offer an effective alternative when visual assessment is not possible. Compared to the visual assessment, which largely suffers from bias

and subjectivity when no clear marks are visible, our method offers a non-biased approach. Sometimes, all that is left in the field are mere crumbles of clay with no recognizable imprints (Figure 4). The interpretation thereof is highly speculative, sometimes interpreted as arthropod 'attacks' or simply excluded from the analyses, while potentially containing valuable information (Rößler et al., 2019). Our eDNA-clay protocol is particularly useful in significantly reducing loss of data from unidentifiable attacks (from 28% unidentified in visual assessment down to 15% unidentified in the genetic assessment of the same samples), which is especially valuable when overall attack rates are low. However, as mentioned above, eDNA analysis is prone to false positives. DNA-based results should thus be carefully interpreted and reassessed with bite marks wherever possible. Additionally, and ideally, the ecological validity of the interaction between studied predator and prey species should be cross-validated, for example via faecal eDNA or gut content analysis.

## 4.3 | Further developments

We focused on mammalian predators here for two reasons: First, they are known to attack European fire salamanders in the wild (Seidel & Gerhardt, 2017). Secondly, mammalian bite marks can be visually identified to relatively low taxonomic levels, providing us with a baseline comparison for our analysis. However, many clay model studies focus on predation by non-mammalian, visually oriented predators such as birds (Roslin et al., 2017). Peck marks are not necessarily suitable for species identification and thus remain largely unidentified. This represents a significant knowledge gap for systems where birds are drivers of the evolution of anti-predator coloration and patterns in prey. Compared to a large mammal, birds will likely leave lower amounts of DNA on the model. However, considering the sensitivity of amplification-based eDNA methods, our protocol should be well-suited for the analysis of avian peck marks. Avian DNA was already

successfully isolated from residual saliva in fruits (Monge et al., 2020) and our analysis using mammal-specific primers already identified an attack by a bird of prey. Using avian-specific primers, one should thus be able to readily recover avian DNA.

Beyond predator–prey studies, the eDNA-clay protocol is also suitable for monitoring invasive species. For example, in a study on predation on European fire salamanders on the island of San Martiño (*Salamandra salamandra gallaica*; Velo-Antón & Cordero-Rivera, 2011), bite marks from clay models supported the presence of and predation by non-native invasive rats *R. rattus*, posing an acute threat to the salamander population. Implementing the eDNA-clay protocol could strengthen invasive species monitoring and facilitate subtle but significant species differentiations. Similarly, eDNA in conjunction with clay models may be suitable to support species inventories, particularly targeting nocturnal, primarily fossorial or arboreal species that are difficult to detect using camera traps (Aylward, Sullivan, Perry, Johnson, & Louis, 2018). Lastly, our method has also been suggested in a conservation context, where it could help assess and identify predators potentially targeting vulnerable species during planned reintroductions (Umbers et al., 2020).

5 | CONCLUSIONS

Clay model studies are easy to use, cheap to produce and applicable to a wide range of ecological questions. However, the method largely lacks standardization, particularly when it comes to taxonomic identification of attackers. This problem can be solved by including genetic sampling from bite marks. Our eDNA protocol is an efficient way to identify attacker species and will greatly improve the explanatory power and applicability of clay models for ecological and evolutionary studies. Future research should focus on further exploring eDNA longevity on clay models and broadening the protocol's applicability for a variety of predator taxa.
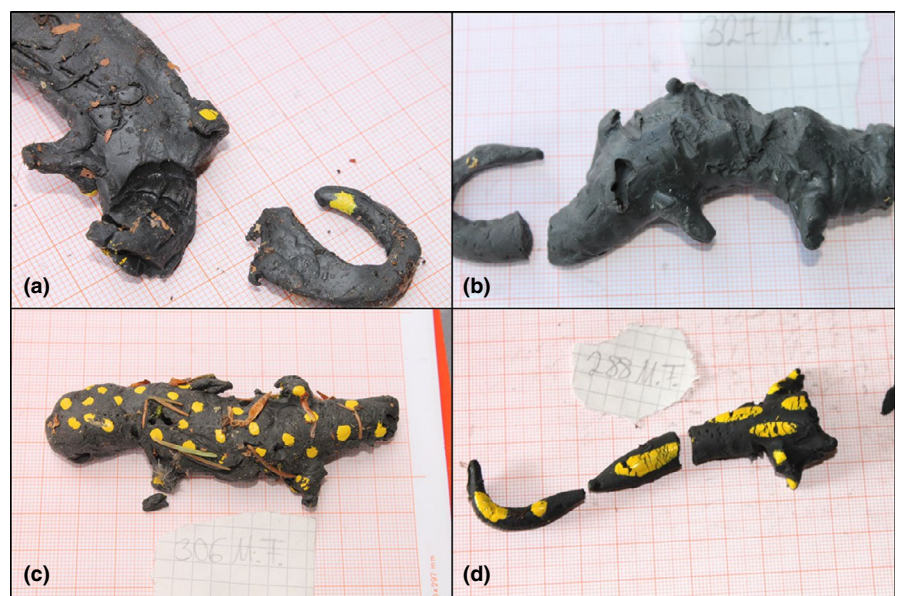
FIGURE 4 Examples of attack marks and their identification: (a) was visually identified as wild boar, (b) as red fox, both were confirmed in the genetic analysis. (c) and (d) could not be identified visually, but turned out to be avian (*Buteo buteo*, c) and red fox (d) in the molecular analysis

AUTHORS' CONTRIBUTIONS

D.C.R. and H.K. wrote the manuscript with support by S.L. and M.V.; D.C.R., S.L. and M.V. conducted the study design; C.P. and M.F. conducted field and laboratory work; additional laboratory analyses were conducted by S.K.; H.K. and M.F. devised the molecular analysis and analysed the molecular data.

COMPETING INTERESTS

The authors declare they have no competing interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

All reads and OTU sequences from this study are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.vdncjsxrk (Rößlerd et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.