

# DNA metabarcoding to assess diet partitioning and feeding strategies in generalist vertebrate predators: a case study on three syntopic lacertid lizards from Morocco

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DNA metabarcoding is a fast and simple alternative to traditional microscopy methods, which have been the main tool for identification of prey in dietary studies of lizards. In this study, we applied a metabarcoding approach based on *COI* and 16S rRNA amplicons to assess diet partitioning and feeding strategies in three syntopic lizards from Taza, Morocco: *Scelarcis perspicillata chabanaudi*, *Scelarcis perspicillata pellegrini* and *Podarcis vaucheri*. In order to avoid competition, these lizards are expected to consume different prey species because they occupy distinct trophic niches, use different foraging strategies and express different dorsal pigmentation patterns. Given the spotted pattern of *S. p. chabanaudi*, we hypothesize a sit-and-wait foraging strategy with a less diverse diet and a higher consumption of mobile prey relative to the striped *S. p. pellegrini* and *P. vaucheri* which, as potential active foragers, are expected to have a higher diet diversity. Previous diet assessments using microscopy on faecal remains seem to contradict these expectations. Our results show that, as expected, the diet of *S. p. chabanaudi* is less diverse than the diet of *S. p. pellegrini*. Regarding *P. vaucheri*, our dietary data are consistent with the hypothesis that this species behaves as an active forager, owing to its high niche overlap with *S. p. pellegrini*. Advantages and limitations of molecular barcoding compared with the microscopy approach to the analysis of lizard diets are discussed.

ADDITIONAL KEYWORDS: 16S rRNA – cytochrome *c* oxidase subunit I – diet analysis – *Podarcis vaucheri* – *Scelarcis perspicillata* – trophic niche partitioning.

## INTRODUCTION

Despite most lizards being considered generalists (Díaz & Carrascal, 1990), feeding habits are expected to vary among species depending on the foraging strategy adopted and on prey availability, mobility, behaviour, size or hardness (Vanhooydonck *et al.*, 2007).

Dietary studies on lizards have traditionally relied on morphological prey identification in stomach contents obtained through animal sacrifice (e.g. Díaz & Carrascal, 1990) or stomach flushing (e.g. Luiselli *et al.*, 2011), or by using faecal samples (e.g. Perera *et al.*, 2006). These methods require considerable

taxonomic expertise, because prey items are identified from undigested partial remains, which can also limit the detection of soft prey owing to their high digestibility (Pompanon *et al.*, 2012; Taberlet *et al.*, 2012). In fact, it is suggested that the absence of trophic niche structure found for many lizards can be attributable to difficulties in identifying prey taxa at a finer scale than the typical order or family (Luiselli, 2008a), which can be caused by an oversimplification of the diversity of consumed prey, especially in taxonomically complex groups (e.g. Perera *et al.*, 2006; Hawlena & Pérez-Mellado, 2009).

Currently, we are facing a so-called 'taxonomic impediment', caused by a shortage of taxonomy experts, which coincides (or not) with the rise in popularity of

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molecular barcoding methods. These approaches allow family-, genus- or species-level identifications, because there is an accurately curated barcode database (e.g. [Jeanniard-du-Dot et al., 2017](#); [Buglione et al., 2018](#)). At present, DNA metabarcoding is a fast and simple alternative to traditional microscopy methods, because it allows the detection of prey DNA in digested samples and has been shown to be effective in recovering previously undetected prey groups (e.g. [Jarman et al., 2013](#); [Sousa et al., 2016](#)). Nevertheless, studies relying on metabarcoding need to be designed carefully, because taxonomic biases can arise related to primer choice ([Piñol et al., 2015](#)). The mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene is the standard marker in animal barcoding owing to the availability of extensive taxonomically verified databases and its easy amplification and higher substitution rate compared with other genes, such as ribosomal RNA (rRNA), which improves taxonomic resolution ([Yu et al., 2012](#)). Its high mutation rate, however, constitutes a problem when using metabarcoding owing to the difficulty in designing universal primers ([Deagle et al., 2014](#)), especially for genetically diverse groups, such as insects ([Clarke et al., 2014](#)). For this reason, more conservative markers, such as the 16S rRNA, have been proposed and used to design truly universal primers, thereby enabling the detection of more taxa (e.g. [Elbrecht et al., 2016](#)).

In this study, we applied a molecular barcoding approach to assess diet partitioning and feeding strategies in three syntopic lacertid lizards from north-east Morocco. The Moroccan rock lizard, *Scelarcis perspicillata* (Duméril & Bibron, 1839), is a small lacertid inhabiting Morocco and western Algeria ([Bons & Geniez, 1996](#)). It is a very agile lizard and an excellent climber, feeding mainly on beetles, ants, spiders, flies and small moths ([Schleich et al., 1996](#); [Perera et al., 2006](#)). Two of the subspecies described, *S. p. chabanaudi* and *S. p. pellegrini*, live in strict syntopy in Taza, a locality in north-east Morocco, and belong to two genetically distinct lineages showing different size and dorsal pattern ([Harris et al., 2003](#)). *Scelarcis p. chabanaudi* is the largest form (mean snout–vent length = 61 mm) and has a dorsum with light spots on a black background, whereas *S. p. pellegrini* is smaller (mean snout–vent length = 54 mm) and presents two light dorsolateral stripes on a spotted dorsum ([Bons & Geniez, 1996](#); [Schleich et al., 1996](#)). In this locality, these two lizards share habitat with another small Mediterranean lacertid, *Podarcis vaucheri* (Boulenger, 1905). This species, which resembles *S. p. pellegrini* both in body size and in pigmentation pattern ([Schleich et al., 1996](#)), is distributed throughout the Northern Maghreb and Southern Iberia ([Kaliontzopoulou et al., 2011](#)) and is considered a food generalist ([Schleich et al., 1996](#)).

A previous diet study of *S. p. pellegrini* and *S. p. chabanaudi* using microscopy on faecal remains showed greater differences in the diet composition of the two forms when living in syntopy in Taza than in allopatry, which might indicate the use of different foraging strategies to reduce intraspecific competition ([Perera et al., 2006](#)). Two extreme foraging strategies within a continuum are recognized for lizards: active foraging and sit-and-wait ([Pianka, 1966](#); [Perry, 1999](#)). Active foragers move throughout extensive areas looking for prey and are more likely to find sedentary, clumped and unpredictably distributed prey, such as insect larvae or termites. On the contrary, sit-and-wait predators wait for prey in a stationary place and quickly attack them when they approach, thus tending to eat more active and mobile prey ([Huey & Pianka, 1981](#); [Verwajen & Van Damme, 2007](#)). Foraging modes are often associated with dorsal pigmentation, which is also known to play an important role in escape from predators ([Jackson et al., 1976](#)). Cryptic body coloration patterns reduce the probability of detection by predators because they blend with the natural background, working as a camouflage mechanism ([Halperin et al., 2017](#)). Conversely, conspicuous pigmentation, such as stripes, seemingly disadvantageous in most environments, can perform well during movement ([Stevens et al., 2011](#)), because longitudinal stripes and high-contrast patterns interfere with the perception by the predator of the speed and trajectory of the moving prey, creating a ‘motion dazzle’ phenomenon ([Jackson et al., 1976](#)). This leads to an associated evolution of foraging behaviour and morphology, in which active foraging modes are usually associated with striped dorsal patterns, whereas sit-and-wait predators present cryptic pigmentation ([Halperin et al., 2017](#)). In our case study, *S. p. chabanaudi* fits the phenotype that is generally expected for a sit-and-wait forager, whereas *S. p. pellegrini* and *P. vaucheri* phenotypes would fit what is expected for an active forager. However, previous diet analysis by [Perera et al. \(2006\)](#) seemingly contradicted this association of phenotype and foraging strategy. However, their study was based on microscope diet analysis and, as such, the dietary spectrum and the degree of dissimilarity between the diets of *S. perspicillata* forms found at Taza might be underestimated owing to the low taxonomic resolution of the methodology used.

In the present study, we used a metabarcoding approach based on partial *COI* and 16 rRNA gene sequences to study the diets of *S. p. chabanaudi* and *S. p. pellegrini* in Taza with two main objectives: (1) to determine whether molecular barcoding allows for an increased taxonomic resolution of diet analyses relative to the microscopy approach; and (2) to assess whether the diet partitioning detected by [Perera et al. \(2006\)](#) between these two forms is part of a consistent pattern

linked to different foraging strategies associated with their dorsal pattern of pigmentation. Additionally, we also examined the niche overlap of the two *S. perspicillata* forms with the more generalist lizard *P. vaucheri* (Boulenger, 1905). Given that the colour pattern of this lizard resembles that of *S. p. pellegrini*, we hypothesize similar foraging strategies.

## MATERIAL AND METHODS

### SAMPLING

Samples were collected in September 2016 at Taza (34°12'94"N, 4°3'14"W), in north-eastern Morocco. This area is dominated by *Quercus* trees, shrubs and large rock outcrops. A total of 68 adult individuals were included in the study, of which 25 were *S. p. chabanaudi*, 28 *S. p. pellegrini* and 15 *P. vaucheri* (Supporting Information, Table S1). Lizards were captured with a noose while active on trees, rocks and on the ground. All animals were identified, sexed (following Perera *et al.*, 2006, 2007), measured (snout–vent length) to the nearest millimetre, and individual pellets were collected and preserved in 96% ethanol. After data collection, all individuals were released unharmed at the point of capture.

### PRIMER CHOICE AND BLOCKING OLIGONUCLEOTIDE DESIGN

A pilot study was performed to test published primers targeting regions within the *COI* and 16S rRNA genes in order to find the most suitable primer pair for each gene. The most-consumed prey reported from previous studies (Carretero *et al.*, 2006; Perera *et al.*, 2006) represented five invertebrate orders (Coleoptera, Orthoptera, Diptera, Odonata and Hemiptera), and species belonging to these groups were used to test the efficacy of different primer pairs. Additionally, lizard faecal samples from previous expeditions were also used in these tests. Host DNA from *S. p. chabanaudi*, *S. p. pellegrini* and *P. vaucheri* was also included for a preliminary evaluation of host co-amplification. The primer pairs Ins16S\_1F/Ins16S\_1R (5'-TRRGACGAGAAGACCCTATA-3'/5'-TCTTAATCCAACATCGAGGTC-3'; Clarke *et al.*, 2014) targeting 16S and mlCOIintF/jgHCO2198 (5'-GGWACWGGWTGAACWGTWTAYCCYCC-3'/5'-TAIACYTCIGGRTGICCRARAAYCA-3'; Leray *et al.*, 2013) targeting *COI* were the primers with higher amplification success covering all the tested taxa groups and were therefore chosen for the present study.

Based on the positive results of host amplification with the selected primers, blocking primers were designed following Vestheim & Jarman (2008) to

minimize host amplification. One single blocking primer was used for the amplification of the 16S fragment (5'-CGAGGTCGTAAACCCCTTGTCGATA GGG[SpC3]-3') for the three study species, whereas for the *COI* two different blocking primers were designed for *S. p. chabanaudi* and *S. p. pellegrini* (5'-GTCTAC CCACCTTTAGCTGGC[SpC3]-3' and 5'-GTTTATCC GCCTTTAGCTGGC[SpC3]-3', respectively). We did not use any host DNA removal strategy for *P. vaucheri* owing to the lack of published *COI* sequences essential to design the blocking oligonucleotide.

### DNA EXTRACTION

DNA extraction was performed in a positive controlled pressure room designed to prevent contamination. Before extraction, samples were dehydrated at 37 °C overnight. When more than one pellet per individual was available, these were pooled to increase the probability of detecting the highest number of prey (Pompanon *et al.*, 2012). Extraction was performed using the PureLink Genomic DNA kit (Thermo Fisher Scientific) following the manufacturer's protocol, with triple volumes of lysis buffer and proteinase K to improve DNA extraction. All samples were vortexed to disrupt the faecal mass and digested overnight. Extracted DNA was stored at –20 °C until preparation of the library.

### LIBRARY PREPARATION AND SEQUENCING

A two-step polymerase chain reaction (PCR) approach was performed, following Kozich *et al.* (2013). For the first PCR step, the 16S rRNA fragment was amplified with a 15× blocking oligonucleotide concentration relative to the PCR primers. A touchdown PCR protocol was implemented, starting at 65 °C annealing temperature and decreasing by 0.5 °C per cycle until 55 °C, followed by 25 cycles at this temperature. For the *COI* fragment, PCR primers were combined with a 20× blocking primer concentration. A touchdown PCR was also performed, with the annealing temperature decreasing by 1 °C each cycle from 67 to 46 °C and then amplification for 25 cycles. All PCRs contained bovine serum albumin (25 mM), and the optimal MgCl<sub>2</sub> concentration was 2.5 mM. Platinum Taq Polymerase (Invitrogen; 2 U/μL) was mixed in a 10 μL reaction volume with 0.5 μL of DNA. The PCRs were run in triplicate, with a negative control (blank), a positive control (prey sample) and a host sample, to check the effectiveness of the blocking primer. Amplified fragments were tested in 2% agarose gel and then replicate PCRs were pooled. PCR purification was performed using the Agencourt AMPure XP (Beckman Coulter) system with a proportion of 0.8 μL of magnetic



beads to 1  $\mu$ L of PCR product. This ratio allowed the removal of primer-dimer and unincorporated reagents.

In order to attach a unique tag to each sample in addition to Illumina sequencing adaptors, a second PCR was performed in a 10  $\mu$ L reaction volume using the same conditions as the first PCR at 55 °C annealing temperature for ten cycles. A final purification was performed using the ratio of 1.2  $\mu$ L of beads to 1  $\mu$ L of PCR. Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) was used to quantify the amplicon concentration, and all samples were normalized to 15 nM and then finally pooled with 2  $\mu$ L of each sample.

Sequencing was outsourced to a commercial company (Genewiz) and conducted in an Illumina MiSeq sequencer with 2  $\times$  250 bp paired-end configuration, and  $\leq$  30% of PhiX was spiked-in to increase sequencing diversity. Demultiplexed sequences were deposited at sequence read archive.

#### BIOINFORMATIC ANALYSIS AND TAXONOMIC ASSIGNMENT

Samples were de-multiplexed, and adaptors were removed by the sequencing company. From this step on, *COI* and 16S samples were processed independently. Fastq files were analysed using USEARCH v.9.2.64 (Edgar, 2010), where reads were assembled, quality filtered and clustered into operational taxonomic units (OTUs).

First, the overall sequencing quality was checked, and the number of reads, expected error (EE) and length distribution were summarized for forward (R1) and reverse (R2) reads separately. After quality control, primers were removed, and paired-end reads were assembled using the command *-fastq\_mergepairs*. Samples were filtered by quality with the *-fastq\_filter* command, which was first set to discard reads shorter than 100 bp (Yu *et al.*, 2012) and then to filter them by overall quality scores. The maximal expected sequencing error was set to one owing to the overall good sequencing quality (Edgar & Flyvbjerg, 2015). Then *-fastx\_uniques* was set to discard replicated reads and singletons (sequences represented by a single read). Unique sequences were clustered into OTUs using the UPARSE algorithm (Edgar, 2013) with *-cluster\_otus*. In this step, chimeric sequences were discarded, and each sequence should match with one OTU with  $\geq$  97% identity. Lastly, *-usearch\_global* was used in order to create an OTU table with the frequency of all OTUs per sample. For each sample, prey detection was normalized by removing OTUs representing  $<$  0.5% of the total number of reads of that sample (Deagle *et al.*, 2019).

Sequences from 16S rRNA were compared against the National Center for Biotechnology Information (NCBI) database using the BLAST algorithm, and

sequences from *COI* were compared with both NCBI and BOLD databases. Sequences were identified to family, genus or species when similarities were  $>$  90,  $>$  98 and  $>$  99%, respectively. When equal similarity to two or more taxa was found, sequences were identified to the higher taxonomic level that included both taxa. Sequences with 85–89% similarity were classified to order, and when a match up to the order level was not found, OTUs were considered unidentified. After removal of non-food sequences from the *COI* dataset, 45% of the samples ended up with  $<$  100 sequences per sample. Owing to this, we considered this dataset not representative enough for each species, and these results were not analysed further.

#### DIET ANALYSIS AND STATISTICS

As mentioned above, the *COI* dataset was not analysed statistically; therefore, the following details of methods apply only to the 16S dataset. The frequency of occurrence of each prey was calculated by dividing the number of faecal samples containing a certain food item by the total number of faecal samples for each lizard species (Deagle *et al.*, 2019).

Diet diversity was determined for each species using Levin's niche breadth index, with the *spaa* package in R software (Zhang, 2016). Niche overlap was calculated for the community and between pairs of species through Pianka's niche overlap index, with the frequency of occurrence of each prey at the order, family and OTU level so that the sensitivity of this index could be tested at different scales. The observed overlap values were compared with a distribution of expected overlap values based on null model simulations using the package *EcoSimR* (Gotelli *et al.*, 2015). The expected distribution resulted from a simulation with 10 000 randomizations using the RA2 and RA3 algorithms. RA2 substitutes a random number of niche breadth utilizations, retaining the zero structure of the matrix. On the contrary, RA3 retains the niche breadth of each species and reshuffles the matrix structure given by the zero structure of the resources. Although RA2 had been suggested to be better than RA3 in detecting non-random structure along the trophic niche dimension in lizard communities (Luiselli, 2008a), the latter was also used in order to test the effect of retaining the zero structure of the data. The observed overlap value was considered statistically different from the null model when  $P_{\text{obs} < \text{exp}} <$  0.05, with either RA2 or RA3.

Statistical differences among the species were computed using the *vegan* (Oksanen *et al.*, 2018) package in R software. The OTU table on the presence of items consumed was transformed into a distance matrix using the Jaccard index. Statistical significance was determined with a non-parametric permutational analysis of variance (perMANOVA) among species

and sex using 1000 random permutations. Post hoc pairwise perMANOVA comparisons were performed with Bonferroni correction to determine which pair of species contributed to the differences observed.

In order to confirm the precision of our results, the effect of sample size was tested on the pseudo-multivariate dissimilarity-based standard error (MultSE) using the R function provided by Anderson & Santana-Garcon (2015).

All analyses were performed in the R environment (RStudio Team, 2016; R Core Team, 2017), and statistical significance was set to  $P < 0.05$ .

## RESULTS

### ANALYSIS OF SEQUENCES

From the 68 samples collected, 53% ( $N = 36$ ) were successfully amplified with the 16S primers and 69% ( $N = 47$ ) with the *COI* primers. Assembling forward and reverse reads produced 575 679 sequences for 16S and 752 611 for *COI*, of which 44 567 and 97 981 unique sequences, respectively, were retained.

Regarding 16S sequences, one sample of *S. p. pellegrini* was removed after quality filtering. Moreover, another sample from *S. p. chabanaudi* whose diet was mainly constituted by *Androctonus gonneti* DNA was also removed owing to a strong suspicion that it was a laboratory contaminant, because this scorpion species is exclusive to desert areas from Africa (Lourenço & Qi, 2007) and the haplotype was identical to one amplified previously in the same laboratory. Sequence clustering resulted in a total of 84 identified OTUs, of which 18% belonged to host, protists and sequences considered unidentified. After the removal of OTUs representing  $< 0.5\%$  of each sample, a total of

45 OTUs identified to the order level were recovered. Of those, 33 could be assigned to the family, 13 to the genus and five to the species.

The *COI* dataset was not analysed in such detail because a low number of prey sequences per sample was retrieved after taxonomic identification.

### DIET COMPOSITION

Globally, the most common identified prey with the 16S rRNA marker was Coleoptera (present in 19 individuals, 56%), followed by Hymenoptera (16 individuals, 47%). Within the Hymenoptera order, Formicidae (ants), which was considered different from the other Hymenoptera families given their clumped habits, was present in 44% of the samples. The other consumed prey, although less frequent, belong to the orders Blattodea, Diptera, Hemiptera, Lepidoptera and Orthoptera (Fig. 1). Interestingly, *COI* sequences revealed the additional presence of Arachnida, such as Araneae and Sarcopiformes (mites), although its frequency of occurrence was not calculated owing to the amplification of a high number of non-target sequences with this marker, resulting in a reduced dataset size for each species. Moreover, sequences classified as molluscs were not considered as prey because it was not possible to assign them to a higher taxonomic classification.

*Scelarcis perspicillata pellegrini* was the species with the most diverse diet, with the presence of 28 different OTUs belonging to 12 prey families and seven orders (Fig. 2). This species presented the highest niche width values (4.703 within the order level, 5.765 within family and 16.714 within OTUs; Table 1), and their diet was mainly composed of Formicidae (56%) and Curculionidae (38%). Representatives of

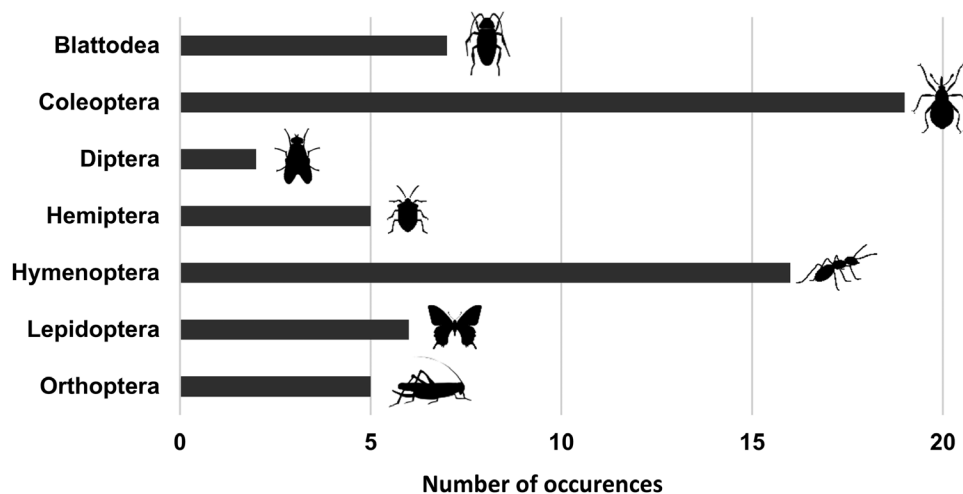
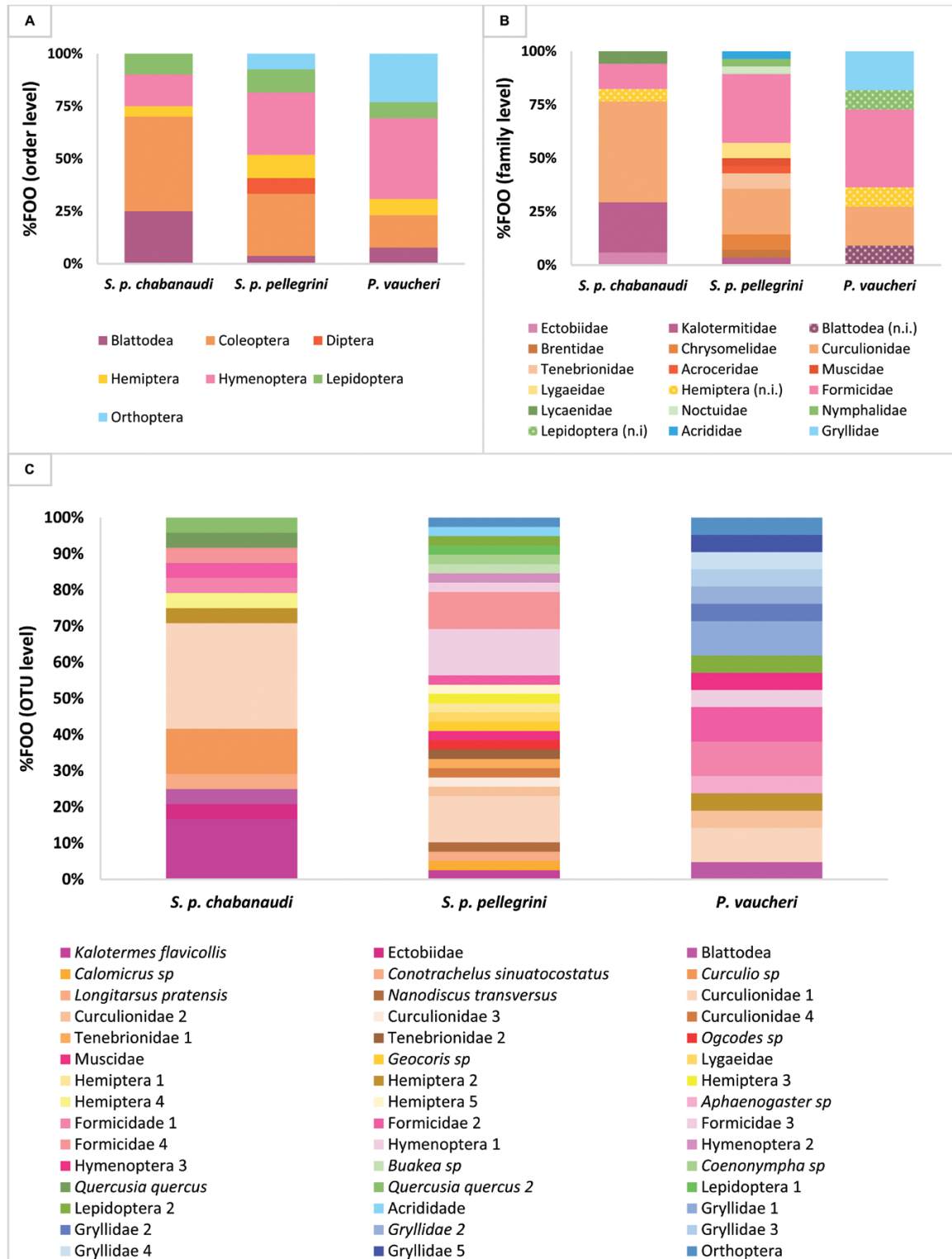


Figure 1. Number of occurrences of prey items for the 16S marker among all samples.



**Figure 2.** Percentage frequency of occurrence of prey items found for *S. p. chabanaudi*, *S. p. pellegrini* and *P. vaucheri*. A, the identified orders. B, the identified families. C, the identified operational taxonomic units (OTUs) Abbreviation: n.i., not identified.

**Table 1.** Levin's index to calculate niche width for each species at the order, family and operational taxonomic unit level

	Niche width (Levin's index)		
	Order	Family	Operational taxonomic unit
<i>S. p. chabanaudi</i>	3.333	3.322	6.857
<i>S. p. pellegrini</i>	4.703	5.765	16.714
<i>P. vaucheri</i>	4.122	4.481	15.207

the Chrysomelidae family (beetles) were present in two *S. p. pellegrini* samples, one of which was identified as *Longitarsus* sp. (flea beetle). In the diet of *S. p. chabanaudi*, 13 OTUs were detected, which belong to six different families and five orders. Its niche width was the lowest among the three studied species (3.333 within order, 3.322 within family and 6.857 within OTUs; [Table 1](#)). Curculionidae (weevils) and Kalotermitidae (termites) were the most consumed prey, with a frequency of occurrence of 62 and 31%, respectively ([Fig. 2](#)).

Among *P. vaucheri*, 57% of the individuals consumed Formicidae, which was the prey with the highest frequency of occurrence. Its niche width was very similar to the one of *S. p. pellegrini* (4.122 within order, 4.481 within family and 15.207 within OTUs; [Table 1](#)). Ectobiidae (cockroaches) and Gryllidae (crickets), which are comparably larger prey than all the other identified prey, were present only in the diets of *S. p. chabanaudi* and *P. vaucheri*, respectively.

Niche overlap considering order- and family-level prey was higher between *S. p. pellegrini* and *P. vaucheri*, whereas the lowest overlap was found for *S. p. chabanaudi* and *P. vaucheri* ([Table 2](#)). However, those were not statistically significant when using the order level. When OTUs were used to calculate Pianka's index, the highest niche overlap was detected between *S. p. chabanaudi* and *S. p. pellegrini* ([Table 2](#)).

The perMANOVA showed no significant interaction effect between species and sex (d.f. = 1; residual d.f. = 29;  $R^2 = 0.021$ , pseudo- $F = 0.713$ ,  $P = 0.846$ ). Differences among the diet of the three studied species were statistically significant (d.f. = 2; residual d.f. = 29;  $R^2 = 0.103$ , pseudo- $F = 1.778$ ,  $P = 0.004$ ), but no differences between sexes were found (d.f. = 1; residual d.f. = 29;  $R^2 = 0.032$ , pseudo- $F = 1.099$ ,  $P = 0.326$ ). Despite the differences found among the three species, pairwise perMANOVA revealed no statistical differences between any species pair (*P. vaucheri* vs. *S. p. chabanaudi*: d.f. = 1;  $R^2 = 0.104$ , pseudo- $F = 1.981$ ,  $P = 0.060$ ; *P. vaucheri* vs. *S. p. pellegrini*: d.f. = 1;  $R^2 = 0.059$ , pseudo- $F = 1.272$ ,  $P = 0.119$ ; *S. p. chabanaudi* vs. *S. p. pellegrini*: d.f. = 1;  $R^2 = 0.076$ , pseudo- $F = 2.069$ ,  $P = 0.060$ ). The power analysis testing the appropriateness of our

sample size to obtain reliable results showed that for *S. p. chabanaudi* and *S. p. pellegrini* the sample size was sufficient. However, conclusions regarding *P. vaucheri* should be taken with caution because its multivariate standard error did not level out with our sample size (Supporting Information, [Fig. S1](#)).

## DISCUSSION

Metabarcoding allowed an OTU (species)-level diet analysis, reinforcing the power of molecular techniques concerning prey identification in lizards ([Kartzinel & Pringle, 2015](#); [Pinho et al., 2018](#)). Regarding marker choice, the preferential amplification of non-target items (e.g. fungi) when using *COI* confirmed the usefulness of the 16S rRNA to study the diet of insectivorous predators and reinforced the idea that primer choice is of utmost importance in diet metabarcoding studies ([Clarke et al., 2014](#)).

Our results were generally concordant with the previous study by [Perera et al. \(2006\)](#) using microscopy; marked differences between the diets of *S. p. chabanaudi* and *S. p. pellegrini* were confirmed by our analysis, although in the opposite direction. A more diverse diet was found for *S. p. pellegrini* than for *S. p. chabanaudi*, contradicting the previous study, but supporting the general expectation for an active forager. In the present study, the diet of *S. p. chabanaudi* was mainly composed of clumped prey, commonly consumed by active foragers, in contrast to the expectations raised by its coloration pattern, suggesting that this form can adopt an active food search. However, whether the high intake of termites and weevils reflects their abundance in Taza or whether they are actively selected remains uncertain without prey availability studies. In contrast, the diet of *S. p. pellegrini* included four flying prey families, whereas for *S. p. chabanaudi* only one was found. This is in accordance with what was reported by [Perera et al. \(2006\)](#) and seems to contradict the general prediction linking predator phenotype to foraging strategy and specific prey consumption in this case. However, flying prey from the orders Lepidoptera, Hymenoptera and Diptera undergo a process of complete metamorphosis, and the



**Table 2.** Pianka's niche overlap values calculated for each species pairwise and for the community at the order, family and operational taxonomic unit level

	Order			Family			Operational taxonomic unit		
	Observed Pianka's index	$P_{\text{obs-exp}}$		Observed Pianka's index	$P_{\text{obs-exp}}$		Observed Pianka's index	$P_{\text{obs-exp}}$	
		RA2	RA3		RA2	RA3		RA2	RA3
<i>S. p. pellegrini</i> – <i>S. p. chabanaudi</i>	0.803	0.148	0.067	0.644	0.001*	0.061	0.503	< $1 \times 10^{-5}$ *	0.046*
<i>S. p. pellegrini</i> – <i>P. vaucheri</i>	0.851	0.139	0.095	0.792	< $1 \times 10^{-5}$ *	0.011*	0.329	0.040*	0.624
<i>S. p. chabanaudi</i> – <i>P. vaucheri</i>	0.612	0.747	0.307	0.516	0.202	0.081	0.407	0.046*	0.084
Community	0.755	0.254	0.042*	0.650	$3 \times 10^{-5}$ *	0.004*	0.413	< $1 \times 10^{-5}$ *	0.026*

Probability of observed value being lower than the null model ( $P_{\text{obs-exp}}$ ) is given for RA2 and RA3 models. \*  $P < 0.05$ .

metabarcoding approach does not allow different life stages to be distinguished. Additionally, *S. p. pellegrini* consumed members of the Chrysomelidae family (beetles), which are very small and jumping beetles that can also fly and are therefore challenging to find and catch, indicating that active search for these prey might occur.

Although niche overlap was statistically significant at the OTU level in all comparisons, we did not consider this level of resolution the best to evaluate overlap owing to our small sample size compared with the high prey diversity detected. In our case study, family level overlaps appear to be the more accurate. Interestingly, trophic niche overlap between *P. vaucheri* and *S. p. pellegrini* was the highest among the three syntopic species. A similar pigmentation pattern plus their predisposition to live in the same types of habitats (Schleich *et al.*, 1996) might underlie their similar diets and possible foraging strategies. Niche overlap between *S. p. pellegrini* and *S. p. chabanaudi* was lower, and this trophic niche partitioning could be a result of their syntopic habitat (Luiselli, 2008b), rather than strictly different foraging modes.

Changes in availability of resources related to interannual and seasonal differences, or changes in predation pressure, land use or competition, in addition to distinct methodology, might underlie the broad differences found between the two studies. However, the higher taxonomic resolution provided by metabarcoding is of major importance, because orders such as Coleoptera contain > 200 families, with thousands of genera and species. The possibility of analysing the consumed prey at the OTU level allowed the detection of differences among the studied lizards. This could explain the differences in niche width found herein between the two species, which were not found by Perera *et al.* (2006).

Even though our metabarcoding approach allowed greater taxonomic resolution and an accurate measure of prey diversity, and in particular, the identification of soft prey, which are otherwise undetectable using traditional faecal/stomach analysis (e.g. termites), these methods present some limitations, primarily owing to the impossibility of assessing prey size, hardness and developmental stage (larvae or adults). For example, in contrast to the work by Perera *et al.* (2006), where flying stages were undoubtedly present in the diet of *S. p. pellegrini*, metabarcoding did not allow adult flying prey to be distinguished from earlier non-flying stages. There are, therefore, still advantages to the use of microscopy. However, there are also other aspects, such as economic costs, to be considered. Several studies have demonstrated how using next-generation sequencing approaches now can be cheaper than classic Sanger sequencing in DNA barcoding (e.g. Sonet *et al.*, 2018). In our experience,



if time costs are factored in, a molecular approach can also be economically viable relative to the theoretically 'cheap' microscopy approach. In much the same way that 'integrative' approaches are often preferred for species descriptions (e.g. Dayrat, 2005), application of both microscopy and molecular approaches to diet studies might become the new norm.

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#### REFERENCES

- Anderson MJ, Santana-Garçon J. 2015. Measures of precision for dissimilarity-based multivariate analysis of ecological communities. *Ecology Letters* **18**: 66–73.
- Bons J, Geniez P. 1996. *Amphibiens et reptiles du Maroc (Sahara Occidental compris), atlas biogéographique*. Barcelona: Asociación Herpetologica Española.
- Buglione M, Maselli V, Ripa D, de Filippo G, Trapanese M, Fulgione D. 2018. A pilot study on the application of DNA metabarcoding for non-invasive diet analysis in the Italian hare. *Mammalian Biology* **88**: 31–42.
- Carretero MA, Vasconcelos R, Fonseca M, Kaliontzopoulou A, Brito JC, Harris DJ, Perera A. 2006. Escape tactics of two syntopic forms of the *Lacerta perspicillata* complex with different colour patterns. *Canadian Journal of Zoology* **84**: 1594–1603.
- Clarke LJ, Soubrier J, Weyrich LS, Cooper A. 2014. Environmental metabarcodes for insects: *in silico* PCR reveals potential for taxonomic bias. *Molecular Ecology Resources* **14**: 1160–1170.
- Dayrat B. 2005. Towards integrative taxonomy. *Biological Journal of the Linnean Society* **85**: 407–417.
- Deagle BE, Jarman SN, Coissac E, Pompanon F, Taberlet P. 2014. DNA metabarcoding and the cytochrome *c* oxidase subunit I marker: not a perfect match. *Biology Letters* **10**: 1789–1793.
- Deagle BE, Thomas AC, McInnes JC, Clarke LJ, Vesterinen EJ, Clare EL, Kartzinel TR, Eveson JP. 2019. Counting with DNA in metabarcoding studies: how should we convert sequence reads to dietary data? *Molecular Ecology* **28**: 391–406.
- Díaz JA, Carrascal LM. 1990. Prey size and food selection of *Psammmodromus algirus* (Lacertidae) in Central Spain. *Journal of Herpetology* **24**: 342–347.
- Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**: 2460–2461.
- Edgar RC. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods* **10**: 966.
- Edgar RC, Flyvbjerg H. 2015. Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics* **31**: 3476–3482.
- Elbrecht V, Taberlet P, Dejean T, Valentini A, Usseglio-Polatera P, Beisel JN, Coissac E, Boyer F, Leese F. 2016. Testing the potential of a ribosomal 16S marker for DNA metabarcoding of insects. *PeerJ* **4**: 1966.
- Gotelli JN, Hart ME, Ellison MA. 2015. *EcoSimR: null model analysis for ecological data. R package version 0.1.0*. Available at: <http://github.com/gotellilab/EcoSimR>
- Halperin T, Carmel L, Hawlena D. 2017. Movement correlates of lizards' dorsal pigmentation patterns. *Functional Ecology* **31**: 370–376.
- Harris DJ, Carretero MA, Perera A, Ferrand N. 2003. Complex patterns of genetic diversity within *Lacerta (Teira) perspicillata*: preliminary evidence from 12S rRNA sequence data. *Amphibia-Reptilia* **24**: 386–390.
- Hawlena D, Pérez-Mellado V. 2009. Change your diet or die: predator-induced shifts in insectivorous lizard feeding ecology. *Oecologia* **161**: 411–419.
- Huey R, Pianka ER. 1981. Ecological consequences of foraging mode. *Ecology* **62**: 991–999.
- Jackson JF, Ingram W III, Campbell HW. 1976. The dorsal pigmentation pattern of snakes as an antipredator strategy: a multivariate approach. *The American Naturalist* **110**: 1029–1053.
- Jarman SN, McInnes JC, Faux C, Polanowski AM, Marthick J, Deagle BE, Southwell C, Emmerson L. 2013. Adélie penguin population diet monitoring by analysis of food DNA in scats. *PLoS One* **8**: e82227.
- Jeanniard-du-Dot T, Thomas AC, Chérel Y, Trites AW, Guinet C. 2017. Combining hard-part and DNA analyses of scats with biologging and stable isotopes can reveal different diet compositions and feeding strategies within a fur seal population. *Marine Ecology Progress Series* **584**: 1–16.
- Kaliontzopoulou A, Pinho C, Harris DJ, Carretero MA. 2011. When cryptic diversity blurs the picture: a cautionary tale from Iberian and North African *Podarcis* wall lizards. *Biological Journal of the Linnean Society* **103**: 779–800.
- Kartzinel TR, Pringle RM. 2015. Molecular detection of invertebrate prey in vertebrate diets: trophic ecology of Caribbean island lizards. *Molecular Ecology Resources* **15**: 903–914.

- Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013.** Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology* **79**: 5112–5120.
- Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V, Boehm JT, Machida RJ. 2013.** A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Frontiers in Zoology* **10**: 34.
- Lourenço WR, Qi JX. 2007.** A new species of *Androctonus* Ehrenberg, 1828 from Mauritania (Scorpionales, Buthidae). *Boletín de la Sea* **40**: 215–219.
- Luiselli L. 2008a.** Do lizard communities partition the trophic niche? A worldwide meta-analysis using null models. *Oikos* **117**: 321–330.
- Luiselli L. 2008b.** Community ecology of African reptiles: historical perspective and a meta-analysis using null models. *African Journal of Ecology* **46**: 384–394.
- Luiselli LM, Ebere N, Pérez-Mellado V, Akani GC. 2011.** Stomach flushing affects survival/emigration in wild lizards: a study case with rainbow lizards (*Agama agama*) in Nigeria. *Amphibia-Reptilia* **32**: 253–260.
- Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlenn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H. 2018.** *vegan: community ecology package. R package version 2.4-6.* Available at: <https://CRAN.R-project.org/package=vegan>
- Perera A, Pérez-Mellado V, Carretero MA, Harris DJ. 2006.** Variation between populations in the diet of the Mediterranean lizard *Lacerta perspicillata*. *Journal of Herpetology* **16**: 107–113.
- Perera A, Vasconcelos R, Harris DJ, Brown RP, Carretero MA, Pérez-Mellado V. 2007.** Complex patterns of morphological and mtDNA variation in *Lacerta perspicillata* (Reptilia, Lacertidae). *Biological Journal of the Linnean Society* **90**: 479–490.
- Perry G. 1999.** The evolution of search modes: ecological versus phylogenetic perspectives. *The American Naturalist* **153**: 98–109.
- Pianka ER. 1966.** Convexity, desert lizards, and spatial heterogeneity. *Ecology* **47**: 1055–1059.
- Pinho C, Santos B, Mata V, Seguro M, Romeiras M, Lopes R, Vasconcelos R. 2018.** What is the giant wall gecko having for dinner? Conservation genetics for guiding reserve management in Cabo Verde. *Genes* **9**: 599.
- Piñol J, Mir G, Gomez-Polo P, Agustín N. 2015.** Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Molecular Ecology Resources* **15**: 819–830.
- Pompanon F, Deagle BE, Symondson WOC, Brown DS, Jarman SN, Taberlet P. 2012.** Who is eating what: diet assessment using next generation sequencing. *Molecular Ecology* **21**: 1931–1950.
- R Core Team. 2017.** *R: a language and environment for statistical computing.* Vienna: R Foundation for Statistical Computing.
- RStudio Team. 2016.** *RStudio: integrated development environment for R.* Boston: RStudio, Inc.
- Schleich HH, Kastle W, Kabisch K. 1996.** *Amphibians and reptiles from North Africa.* Königstein: Koeltz Scientific Publications.
- Sonet G, Pauly A, Nagy ZT, Virgilio M, Jordaens K, Van Houdt J, Worms S, De Meyer M, Backeljau T. 2018.** Using next-generation sequencing to improve DNA barcoding: lessons from a small-scale study of wild bee species (Hymenoptera, Halictidae). *Apidologie* **49**: 671–685.
- Sousa LL, Xavier R, Costa V, Humphries NE, Trueman C, Rosa R, Sims DW, Queiroz N. 2016.** DNA barcoding identifies a cosmopolitan diet in the ocean sunfish. *Scientific Reports* **6**: 28762.
- Stevens M, Searle WTL, Seymour JE, Marshall KLA, Ruxton GD. 2011.** Motion dazzle and camouflage as distinct anti-predator defenses. *BMC Biology* **9**: 81.
- Taberlet P, Coissac E, Pompanon F, Brochmann C, Willerslev E. 2012.** Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology* **21**: 2045–2050.
- Vanhooydonck B, Herrel A, Van Damme R. 2007.** Interactions between habitat use, behavior and the trophic niche of lacertid lizards. In: Reilly SM, McBrayer LB, Miles DB, eds. *Lizard ecology: the evolutionary consequences of foraging mode.* Cambridge: Cambridge University Press, 427–449.
- Verwaijen D, Van Damme R. 2007.** Does foraging mode mould morphology in lacertid lizards? *Journal of Evolutionary Biology* **20**: 1950–1961.
- Vestheim H, Jarman SN. 2008.** Blocking primers to enhance PCR amplification of rare sequences in mixed samples - a case study on prey DNA in Antarctic krill stomachs. *Frontiers in Zoology* **5**: 12.
- Yu DW, Ji Y, Emerson BC, Wang X, Ye C, Yang C, Ding Z. 2012.** Biodiversity soup: Metabarcoding of arthropods for rapid biodiversity assessment and biomonitoring. *Methods in Ecology and Evolution* **3**: 613–623.
- Zhang J. 2016.** *Package 'spaa'.* Available at: <https://cran.r-project.org/web/packages/spaa/spaa.pdf>

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** List of samples for this study.

**Figure 1.** Multivariate dissimilarity-based standard error (MultSE) plot.