

# DNA metabarcoding the diet of *Podarcis* lizards endemic to the Balearic Islands

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## Abstract

Dietary studies are essential to unravel the functioning of ecosystems and ultimately to understand biodiversity. This task, which at first may seem simple, becomes especially complex in those cases of omnivorous species with highly variable diets. In this regard, the emergence of next-generation DNA sequencing methodologies represents a powerful tool to address the problem. Here we implement a high-throughput metabarcoding strategy based on the analysis of four molecular markers aimed at sequencing both mitochondrial (animal prey) and chloroplast (diet plants) genome fragments from fecal samples of two lizard species endemic to the Balearic Archipelago (*Podarcis lilfordi* and *P. pityusensis*) obtained through non-invasive methods. The results allowed for the characterization of their diets with a high degree of taxonomic detail and have contributed a large number of new trophic records. The reported diets are based mainly on the consumption of arthropods, mollusks and plants from a diversity of taxonomic orders, as well as carrion and marine subsidies. Our analyses also reveal inter- and intra-specific differences both in terms of seasonality and geographical distribution of the sampled lizard populations. These molecular findings provide new insights into the trophic interactions of these threatened endemic lizards in their unique and isolated ecosystems.

**Key words:** Balearic archipelago, COI, metabarcoding, *Podarcis lilfordi*, *Podarcis pityusensis*, psbA-trnH, rbcL, trophic ecology.

Assessing trophic interactions is essential in understanding ecosystem functionality (Kartzinel and Pringle 2015). Dietary studies provide key information about both the role of species in food webs and the importance of feeding resources for their demographic viability (Alonso et al. 2014), which in turn can influence ecosystem/species management decisions (Rogers et al. 2004; de Sousa et al. 2019). This knowledge is even more relevant under the current environmental changing scenario due to the anthropogenic perturbation of ecosystems with effects on distribution, abundance, and diversity of both consumers and diet resources (Thuiller et al. 2011). However, unveiling the details of the feeding habits of the species can be complex, particularly when dealing with species with highly diverse diets (De Barba et al. 2014). In this regard, omnivorous species feeding on a variety of animal and plant species pose the greater challenge to our ecological understanding of the consumer–resource interactions (De Barba et al. 2014; da Silva et al. 2019; Tercel et al. 2021). The taxonomic identification of the diversity of food items consumed by animals has so far relied on methodologies that are not suited for revealing the full spectrum of food types from mixed diet (De Barba et al. 2014), and in most cases are based on direct observations of feeding activity or microscopic examination of feces. The former approach is time-consuming and difficult to carry out, while the latter is tedious, requires extensive training, fails to identify a variable proportion of food fragments,

and does not allow the detection of soft resources owing to their high digestibility (Valentini et al. 2009; Pompanon et al. 2012; Taberlet et al. 2012). Molecular approaches such as DNA barcoding have arisen as a promising tool to investigate trophic interactions (Symondson 2002; Jurado-Rivera et al. 2009) that in combination with high-throughput sequencing result in a powerful method to decipher a broad range of associations in complex food webs (Littlefair and Clare 2016). To date, only a few DNA metabarcoding studies implementing high-throughput sequencing have investigated the diet of omnivorous animals (reviewed by Tercel et al. 2021), with a bias towards mammal species (De Barba et al. 2014; Robeson et al. 2017; Bonin et al. 2020).

Here we implement a high-throughput sequencing metabarcoding approach based on non-invasive sampling to explore the trophic interactions in two omnivorous lizard species endemic to the Balearic archipelago: *Podarcis lilfordi* (IUCN Endangered; Pérez-Mellado and Martínez-Solano, 2009a) and *P. pityusensis* (IUCN Near Threatened; Pérez-Mellado and Martínez-Solano 2009b). Both species are sister phylogenetic lineages originated ca. 5.33 Ma ago (Rodríguez et al. 2013; but see Salvi et al. (2021) for an alternative dating) with a non-overlapping distribution across the archipelago, represented by allopatric populations inhabiting coastal islands and islets of Mallorca, Menorca and Cabrera (*P. lilfordi*), and the main islands of Ibiza and Formentera

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and many of their islets (*P. pityusensis*). Food resources in such isolated habitats are usually scarce and unpredictable, which could have driven the evolution from their ancestral insectivorous behavior (Pérez-Mellado and Corti 1993) to their present omnivorous feeding on a broad diversity of prey animals, plants, carrion, marine resources, and even juvenile conspecifics (Pérez-Cembranos et al. 2016). Available dietary studies of Balearic lizards are based on classical approaches such as direct observations in the field (Salvador 1986a), analysis of stomach contents from dead specimens (Pérez-Mellado 1989; Pérez-Mellado and Corti 1993), or visual inspection of fecal contents (Pérez-Cembranos et al. 2016; Santamaría et al. 2020). The taxonomic resolution of the food items reported by these studies rarely reaches detailed levels such as species, genus, or family. Our study aims to overcome the limitations of traditional approaches by taking advantage of the capabilities of advanced DNA sequencing methodologies to characterize in detail the trophic spectrum of these lizard species based on a sampling that encompasses their geographic distribution. Additionally, we intend to assess the existence of regional and temporal variation in terms of trophic resources consumption both at inter- and intra-specific levels.

## Materials and Methods

### Sampling

Fresh fecal samples from 242 lizard specimens (*P. lilfordi*: 140; *P. pityusensis*: 102) were collected between the spring of 2016 and the autumn of 2017 from 17 localities in the Balearic archipelago (Table 1 and Figure 1). Lizards were captured by lassoing at daytime and fresh fecal samples were directly obtained with a gentle abdominal massage and stored in absolute ethanol vials. Lizards were sexed based on their

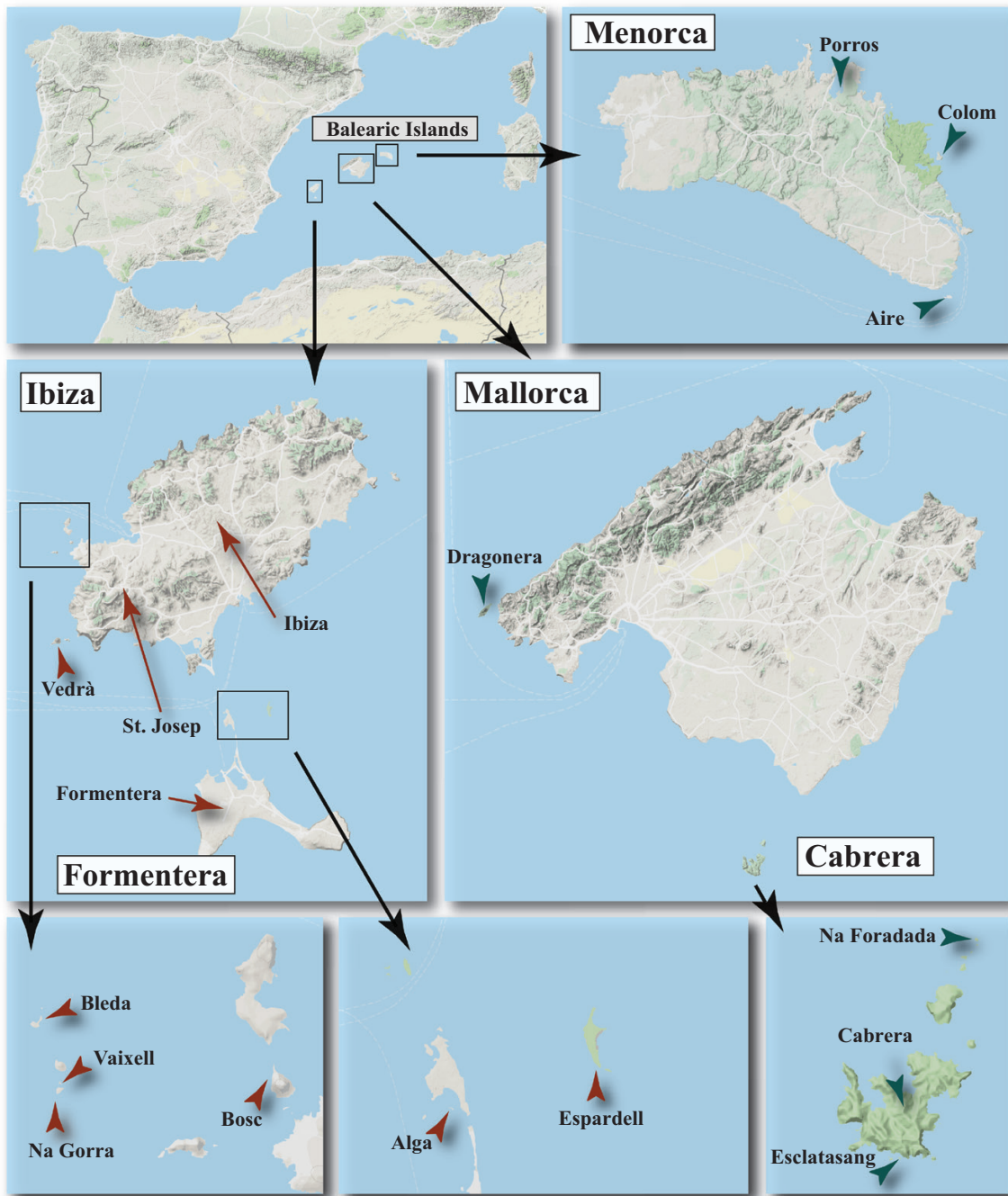
dimorphic attributes (Salvador 2009; Salvador (2009)) and immediately released at the same capture site. Samples were preserved at 4 °C in the field upon arrival to the laboratory and then stored at -20 °C until DNA extraction.

### Molecular analyses

Total DNA was extracted from individual samples using the Isolate Fecal DNA kit (Bioline, London, UK) following the manufacturer protocol and their concentrations were quantified using Qubit Fluorometric Quantitation (ThermoFisher, Foster City, CA, USA). For cost-effective reasons and given that our study seeks to describe the diet composition of each lizard population as a whole, samples from the same population/islet were pooled in equimolar concentrations by sex and collecting year. Samples were submitted to the Roy J. Carver Biotechnology Center (University of Illinois, USA) for amplification of selected markers in a microfluidic high-throughput multiplexed PCR platform (Fluidigm). For animal prey detection we selected two primer pairs: mlCOLintF/dgHCO2198 (5'-GGWACWGGWTGAACWGTWTAYC-CYCC-3'/5'-TAAACTTCAGGGTGACCAARAAY-CA-3'; Meyer 2003; Leray et al. 2013) and ArtF11/ArtR17 (5'-GGNKYNGGNACWGGATGAACWGTNTAYC-CNCC-3'/5'-GGRTCAAAAATGAWGTATTHARAT-TTCGRTCWGTTA-3'; Mallott et al. 2015) targeting the mitochondrial cytochrome oxidase I gene (COI). For diet plant amplification we used two different primer pairs targeting chloroplast DNA regions: the RuBisCO large-subunit gene (*rbcL*) *rbcLa\_F/rbcLa\_R* (5'-ATGTCACCACAAACA-GAGACTAAAGC-3'/5'-GTAAAATCAAGTCCACRCG-3'; Levin et al. 2003; Kress et al. 2009) and the *psbA-trnH* intergenic spacer: *psbA3\_f/trnHf\_05* (5'-GTTATGCATGAACG-TAATGCTC-3'/5'-CGCGCATGGTGGATTCCACAATCC-3'; Sang et al. 1997; Tate and Simpson 2003). CS1 and CS2

**Table 1.** Sampling localities and associated metadata

Species	Localization	Population/ islet	Id	Acronym	Island area (m <sup>2</sup> )	Spring			Summer			Autumn		
						Female	Male	Year	Female	Male	Year	Female	Male	Year
<i>P. lilfordi</i>	CABRERA	Cabrera	Cabrera	C	1.7700.000	-	-	-	12	16	2017	6	7	2016
		Esclatasang	Esclatasang	E	3.900	-	-	-	6	4	2017	4	4	2016
		Na	Na	Fo	12.200	-	-	-	2	8	2017	2	5	2016
		Foradada	Foradada											
	MALLORCA	Dragonera	Dragonera	D	2.880.000	-	-	-	9	8	2016	2	8	2017
	MENORCA	Aire	Aire	A	343.750	5	5	2017	-	1	2016	-	-	-
		Colom	Colom	Co	595.000	6	4	2017	3	3	2016	-	-	-
Porros de Fornells		Porros	Pr	1.600	4	6	2017	-	-	-	-	-	-	
<i>P. pityusensis</i>	FORMENTERA	Alga	Alga	Al	11.250	-	-	-	2/5	3/5	2016/2017	-	-	-
		Espardell	Espardell	Es	561.250	7	6	2017	5	-	2016	-	-	-
		Formentera	Formentera	F	83.240.000	4	6	2017	-	-	-	-	-	-
	IBIZA	Bleda Plana	Bleda	Bp	31.250	-	-	-	-	1	2016	-	-	-
		Bosc de conillera	Bosc	Bc	181.250	4	3	2016	-	-	-	-	-	-
		Na Gorra	Na Gorra	G	15.625	5	5	2017	13	11	2016	-	-	-
		Sant Josep de sa Talaia	St. Josep	St	527.600.000	1	2	2016	-	-	-	-	-	-
		Ses Salines	Ibiza	I	527.600.000	2	2	2016	-	-	-	-	-	-
		Vaixell	Vaixell	Vx	2.604	-	-	-	5	3	2017	-	-	-
		Vedrà	Vedrà	Ve	6.250.000	-	-	-	-	2	2016	-	-	-



**Figure 1.** Maps of the Balearic archipelago showing the location of the sampled *Podarcis lilfordi* (green arrows) and *P. pityusensis* populations (brown arrows). Maps were obtained with Google Maps (Map data 2020 Google) using the function 'get\_map' in the package 'ggmap' version 3.0.0.902 in R version 3.6.3 (See online version for color figure).

Fluidigm universal tags and barcode labels specific to each sample and Illumina adapters i5 and i7 were used and the resulting amplicons were validated on a Fragment Analyzer (Agilent) using the HS NGS kit (DNF-474-33). Sequencing was conducted on an Illumina MiSeq v2 platform yielding  $2 \times 250$  paired-end reads.

Regarding animal prey analyses, a blocking oligo targeting a region within the mitochondrial COI gene was designed to minimize host amplification. We retrieved COI sequences from the most-consumed prey groups reported by previous studies on the diet of the Balearic lizards

(Pérez-Cembranos et al. 2016) available at the Barcode of Life Data System (BOLD; <http://www.boldsystems.org>) and COI sequences from both *P. lilfordi* and *P. pityusensis* available at GenBank (Pérez-Cembranos et al. 2020). DNA sequences were aligned with MAFFT (Katoh and Standley 2013) and used for entropy analysis in BioEdit v. 7.0.5.2 (Hall 1999). Entropy plots revealed a suitable binding site encompassing the 3' end of the mlCOLintF primer annealing site and a conserved sequence region in *Podarcis*. The blocking primer included a C3 spacer at the 3'-end (5'-AACTGTTTACCCCCATTAGCCG[SpC3]-3').

## Sequence analyses and taxonomic assignment

For each marker, Micca version 1.7.2 (Albanese et al. 2015) was used for sequence merging (minimum overlapping region between R1 and R2 reads = 100 nt), trimming (removal of primer sequences and Illumina adaptors), filtering (based on the *filterstats* tool implemented in Micca; maximum error rate = 0.5; chimeric sequences removed) and OTU picking (cut-off value = 0.98). The taxonomic assignment of the inferred OTUs was conducted under a phylogenetic framework. As a first step the resulting DNA sequences (OTUs) were further grouped into 98% similarity clusters with Usearch version 10.0.240\_i860sx32 (Edgar 2010). Usearch was also used to select a representative sequence (centroid) from each cluster that was subsequently used as a BLAST query to retrieve the 1000 most similar sequences from GenBank regardless their respective values of percentage identity. DNA matrices were built for each cluster by combining all dietary sequences in the cluster with the top 1000 hits from its centroid-based BLAST search. The resulting matrices were individually aligned with MAFFT (Katoh and Standley 2013) and used for maximum likelihood phylogenetic inference in IQTREE (Nguyen et al. 2015). Trees were explored in FigTree (Rambaut 2014) to establish the systematic position of diet sequences according to the highest taxonomic rank supported with a nodal bootstrap value  $\geq 70\%$  (Hillis and Bull 1993). Information from multiple markers targeting the same group of organisms (i.e., animals or plants) was merged following the multi-marker metabarcoding approach described in da Silva et al. (2019).

## Molecular marker comparison

The performance of each of the molecular markers selected for our study was assessed based on the frequency of food items reported and identified at different taxonomic ranks (class, order, family, genus, species). Furthermore, the overlap in terms of taxonomic identifications at the above-mentioned taxonomic ranks between markers targeting the same group of dietary organisms (animals or plants) was explored using the R package VennDiagram (Chen and Boutros 2011).

## Datasets

Previous research on the diet of Balearic lizards has demonstrated that food seasonality partially affects diet composition (Pérez-Mellado and Corti 1993; Pérez-Cembranos et al. 2016; Santamaría et al. 2020). In order to assess such seasonal effect and since our dataset did not include samples for all populations and for all seasons of the year due to the remote location of most of the sampled islets and the dependence on meteorological conditions to access them (Table 1), we based our analyses on two different subsets consisting of sampling locations with matching collecting seasons: Spring + Summer dataset (populations: Aire, Colom, Espardell and Na Gorra), and Summer + Autumn dataset (populations: Cabrera, Dragonera, Esclatasang and Na Foradada). In addition, we further explored geographical differences in terms of diet composition by comparing samples from the same collecting season (Spring, Summer and Autumn datasets) either combining the samples from the two lizard species or analyzing each species separately.

## Diet analysis

The weighted percent of occurrence (wPOO) in our dataset was calculated following Deagle et al. (2019), a measure that

considers the percentage of occurrence of each food item across the entire dataset weighted by the total count of prey items detected in each sample. This estimate has been shown to perform better than other indices when investigating taxa with a highly diverse trophic spectrum such as those of the omnivorous Balearic lizards (Andriollo et al. 2019). Niche width at both the species and populational levels was calculated using Levin's index (Levins 1968), whereas trophic niche overlap was estimated based on Pianka's index (Pianka 1973). We tested whether the extent of overlap was greater than expected by chance by comparing the observed values with a distribution of expected overlap values based on null model simulations using the RA3 algorithm (Lawlor 1980) implemented in the package EcoSimR (Gotelli et al. 2015). Differences were considered statistically significant when  $P_{\text{obs} < \text{exp}} < 0.05$ .

To assess whether there are significant differences between the surfaces of the islands and islets inhabited by the two species of lizards, we carried out an unpaired two-samples Wilcoxon test based on the surface data available on the website of the National Institute of Statistics of the Government of Spain ([www.ine.es](http://www.ine.es)) (Table 1). In addition, we conducted Pearson correlation analyses between island area values (Table 1) and the number of different OTUs reported from their inhabiting lizard populations (Supplementary Table S1) to assess if inhabiting larger ranges imply a more diverse trophic niche by predators. Such correlation tests were performed both on the entire sampling and on each of the dataset separated by collecting season (Spring, Summer, Autumn).

Permutational Multivariate Analysis of Variance (PERMANOVA) based on Jaccard distance matrices and 999 random permutations was conducted in the *vegan* R package using the *adonis* function (Oksanen et al. 2007) to test for the effect of the sex, collecting season, and geographical location in resource consumption. The later variable was also explored at a broader scale by assigning each sampling locality (i.e., islet/population) to a main island district in the Balearic archipelago (Mallorca, Menorca, Cabrera, Eivissa or Formentera). Jaccard matrices were also projected through Principal Coordinates Analysis (PCoA) using the R package *phyloseq* (McMurdie and Holmes 2013) to visualize sample distances in terms of diet composition and their associate variables.

Finally, to delve into the objective of assessing the existence of variation in terms of trophic resources consumption at the inter-specific level we tested for differences in the number of diet OTUs per islet/population between lizard species for each of the orders that constitute at least 5% of the diet of either *P. lilfordi* or *P. pityusensis* (animals: Coleoptera, Diptera, Geophilomorpha, Hemiptera, Hymenoptera, Isopoda, Julida, Lepidoptera, Sarcotiformes, Stylommatophora, Trombidiformes; plants: Asterales, Caryophyllales, Pinales, Pottiales). For each consumed order we first conducted a Fisher's *F*-test homoscedasticity analysis to test for the homogeneity of the variances regarding the distribution of consumption frequencies between the populations of the two lizard species. When the variances of both samples were homogenous (Fisher's *F*-test  $P > 0.05$ ) we ran a Student's two-sample *t*-test. By contrast, when heteroscedasticity was present (Fisher's *F*-test  $P < 0.05$ ) we conducted a Welch *t*-statistic. All analyses were carried out with the R package *stats* (R Core Team 2020).

## Results

### Sequence summary

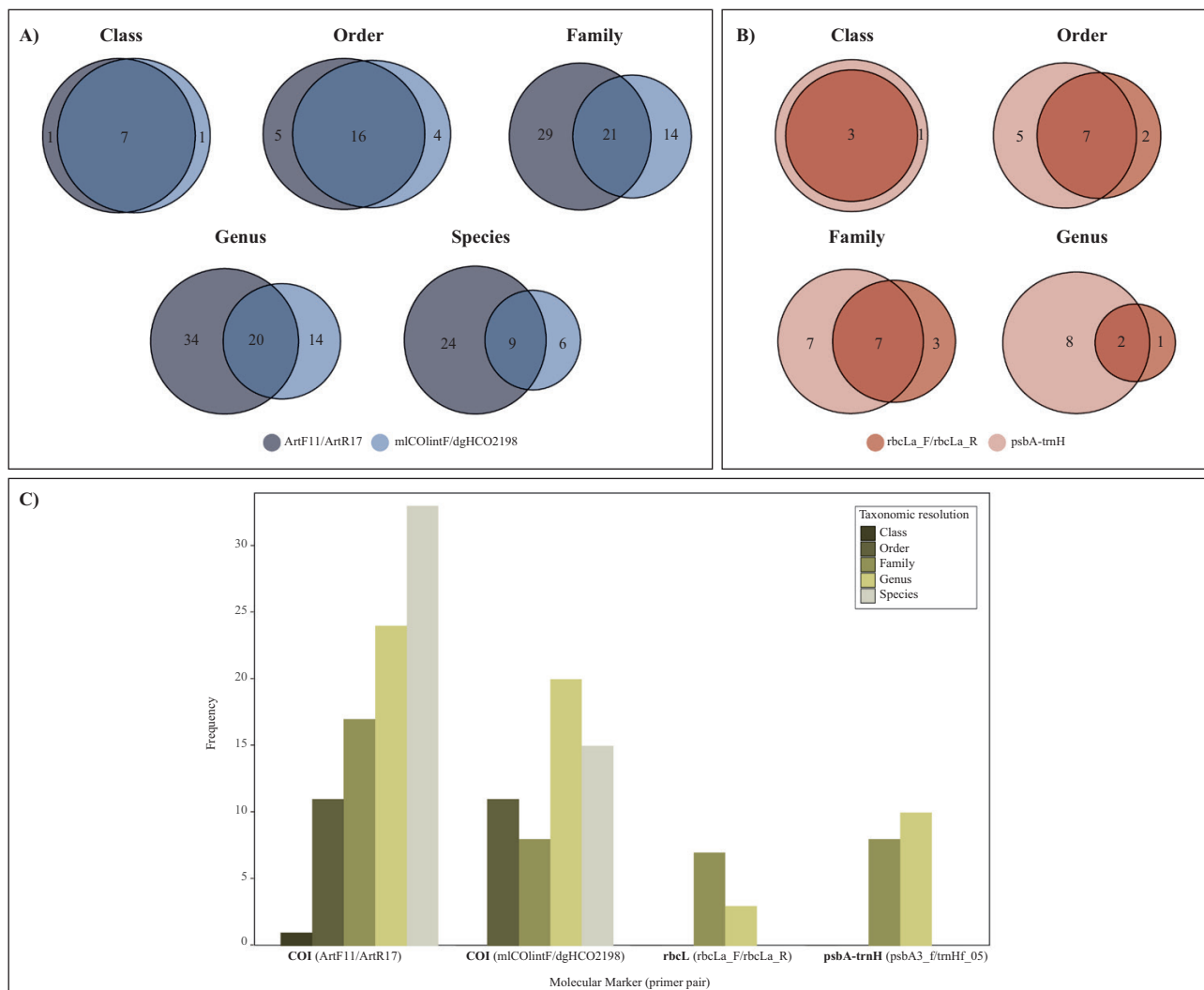
After sequence trimming, merging, and filtering, the final dataset consisted of 989 727 (primer pair ArtF11/Art17), 544 458 (mCIIintF/dgHCO2198), 750 494 (*rbcLa\_F/rbcLa\_R*) and 525 032 (*psbA3\_f/trnHf\_05*) high-quality paired reads. Sequence lengths averaged 236, 313, 228 and 224 bp for the arthropod-specific COI, universal COI, *rbcL* and *psbA-trnH* molecular markers, respectively.

### Differential contribution of molecular markers to diet characterization

There were differences both in terms of the number of OTUs and the accuracy of the identification reported by each of the four primer pairs selected for the study. The arthropod-specific COI primer pair ArtF11/Art17 contributed the highest number of animal prey (86) with most of the identifications reaching the taxonomic level of species (38.4%), followed by genus (27.9%), family (19.8%), order (12.8%) and class (1.1%). Conversely, the universal COI mCIIintF/dgHCO2198 pair yielded fewer OTUs (54) with lower taxonomic accuracy: species (27.8%), followed by genus (37%), family (14.8%),

order (20.4%) and class (0%). Regarding plant-specific markers, the *rbcL* primer pair *rbcLa\_F/rbcLa\_R* retrieved 10 OTUs identified to genus (30%) and family (70%), whereas the oligo combination *psbA3\_f/trnHf\_05* targeting the *psbA-trnH* intergenic spacer contributed more vegetal OTUs (18) with higher taxonomic resolution (genus: 55.6%, family: 44.4%).

Venn diagram analyses and frequency of food items showed the relative contribution of each primer pair in terms of OTU detection at different taxonomic ranks (Figure 2A–C). The two oligo combinations targeting animal prey produced similar results regarding low taxonomic levels (class and order), however the arthropod-specific COI primer pair ArtF11/Art17 outperformed the universal COI pair mCIIintF/dgHCO2198 at high levels where the number of different OTUs contributed exclusively by the former was always higher than the intersection value or the number of OTUs provided by the latter. Such an effect is more noticeable as we go deeper into the taxonomic hierarchy (Figure 2A). The same pattern was observed when comparing the performance of the chloroplast-specific markers, where the *psbA3\_f/trnHf\_05* pair targeting the *psbA-trnH* intergenic spacer provided the largest number of OTUs at all taxonomic levels and showed progressively more



**Figure 2.** Venn diagrams showing the overlap in terms of taxonomic identification of prey animals (A) and diet plants (B) at different taxonomic ranks. (C) Distribution of finest OTU taxonomic identifications (i.e., taxonomic resolution) reported by each of the four molecular markers used in the study.

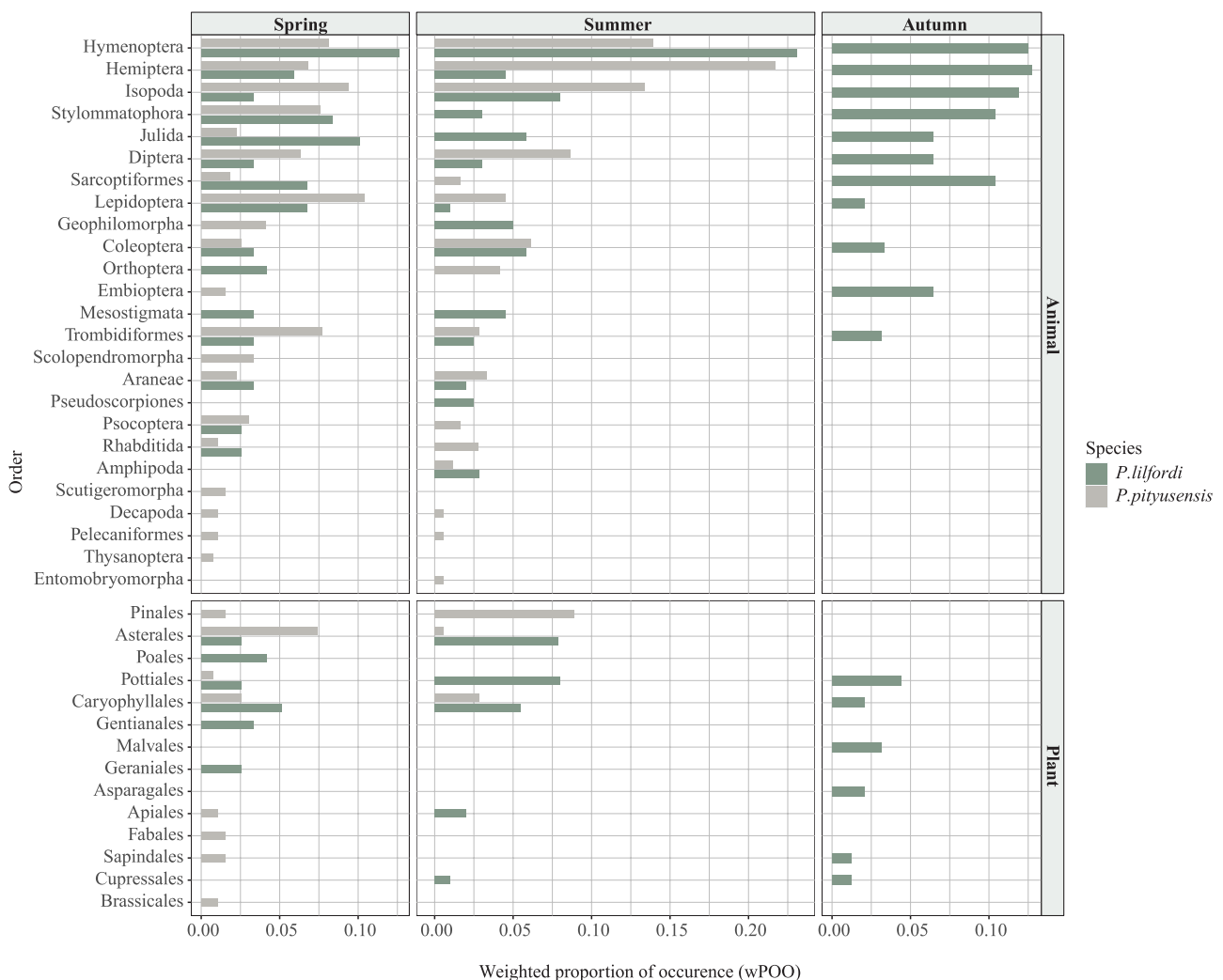
differences against *rbcL* marker the more detailed the taxonomic rank (Figure 2B).

### Diet composition

Balearic *Podarcis* lizards were found to exploit a wide range of food resources. Our metabarcoding approach reported 149 diet taxa of which 124 were prey animal OTUs that could be ascribed to 4 phyla, 9 classes, 25 orders, 64 families, 68 genera, and 39 species (Figure 2 and Supplementary Table S1). Phylum Arthropoda accounted for the highest number of different OTUs (113), followed by Mollusca (9), Nematoda (1) and Chordata (1). Within arthropods, insects resulted to be the most exploited class with 65 distinct OTUs mainly from orders Hemiptera (15), Lepidoptera (15), Hymenoptera (10), Coleoptera (9) and Diptera (7). Arthropod classes Arachnida (25) and Malacostraca (14) were also found to constitute an important fraction of diet of Balearic *Podarcis* lizards. Prey animals from phylum Mollusca were exclusively represented by gastropods from order Stylommatophora (9), while phyla Nematoda and Chordata accounted each for a single representative from orders Rhabditida and Pelecaniformes, respectively. On the other hand, our study revealed the presence in fecal samples

of plant DNA from phylum Streptophyta (25 OTUs) represented by classes Bryopsida (3), Liliopsida (2), Pinopsida (3), and Magnoliopsida (17). Within the latter classes Caryophyllales (6), Apiales (2) and Asterales (3) showed the highest number of different OTUs.

The relative use of trophic resources measured as weighted proportion of occurrence (wPOO) revealed a shared preference for certain food items regardless the lizard species or the collecting season (Figure 3). This was the case of prey animals from orders Hymenoptera, Hemiptera, Diptera, Lepidoptera, Coleoptera, Isopoda and Trombidiformes, and plants from orders Pottiales and Caryophyllales. The fecal samples from *P. lilfordi* showed a consistent feeding throughout the seasons of the year on Hymenoptera, Hemiptera, Isopoda, Stylommatophora, Julida, Diptera, Lepidoptera, Coleoptera and Trombidiformes, as well as plants from orders Pottiales and Caryophyllales (Supplementary Table S1). On the other hand, *P. pityusensis* specimens were found to feed both in spring and summer on Hymenoptera, Hemiptera, Isopoda, Diptera, Sarcoptiformes, Lepidoptera, Coleoptera, Trombidiformes, Araneae, Psocoptera, Rhabditida, Decapoda and Pelecaniformes, and plant items from orders Asterales, Pinales and Caryophyllales (Supplementary Table S1).



**Figure 3.** Relative use of trophic resources measured as weighted proportion of occurrence (wPOO) at the order level per lizard species and season of the year.

### Trophic niche analyses

Levin's indexes for niche breadth showed similar values in *P. lilfordi* samples regardless the collecting season (27.53, 24.30 and 30.72 in spring, summer, and autumn, respectively). In contrast, values for *P. pityusensis* were comparatively high in spring (46.74) and low in summer (13.79) (autumn samples not available for *P. pityusensis*). Regarding Pianka's index of niche overlap (Supplementary Table S2) all pairwise combinations analyzed provided moderate values with an average index of 0.57 ( $SD = 0.11$ , range = 0.33–0.93), although only 5 of such analyses were statistically significant when compared with a null model generated using the RA3 randomization algorithm: comparison between summer samples of *P. lilfordi* and *P. pityusensis* (Pianka's index = 0.526;  $P$ -value = 0.036), and the pairwise analyses between summer samples of Alga and those from Bleda, Vaixell, Vedrà and Espardell (Pianka's indexes = 0.933, 0.785, 0.714, and 0.876, respectively;  $P$ -value < 0.001 in all tests).

### Island size and diet diversity

The unpaired two-samples Wilcoxon analysis to test for differences in terms of island area between the ranges inhabited by both lizard species yielded non-significant results ( $P$ -value = 0.4639). On the other hand, Pearson's correlation analyses also rejected the hypothesis that lizard populations inhabiting larger islands show more diverse diets in terms of number of

different OTUs consumed regardless of whether the analysis was based on the entire sampling (corr. coef = 0.013;  $P$ -value = 0.95) or in the datasets of each season separately (Spring: corr. coef = 0.0032,  $P$ -value = 0.99; Summer: corr. coef = 0.14,  $P$ -value = 0.68; Autumn: corr. coef = 0.84,  $P$ -value = 0.16).

### Variation in the use of trophic resources

PERMANOVA analyses based on samples from the same collecting season found significant differences in terms of diet composition between *P. lilfordi* and *P. pityusensis* in both the Spring and the Summer datasets ( $R^2 = 0.08$ ,  $P$ -value  $\leq 0.004$ ; Table 2). The lizard species variable could not be analyzed in the Autumn dataset due to the lack of *P. pityusensis* samples for this season. These PERMANOVA tests also retrieved as statistically significant the geographical variables defined by both the collecting islet/population ( $R^2$  range = 0.35–0.42,  $P$ -value  $\leq 0.007$ ) and the main island district in the Balearic archipelago to which the sampling site is ascribed ( $R^2$  range = 0.11–0.19,  $P$ -value  $\leq 0.035$ ). Conversely, the sex of the individuals resulted in non-significant results in all tests. On the other hand, PERMANOVA analyses based on samples from populations with matching collecting seasons showed evidence for differences in the use of trophic resources between the two lizard species ( $R^2 = 0.12$ ,  $P$ -value  $\leq 0.001$ ; Table 2), among the source islets/populations ( $R^2$  range = 0.26–0.32,  $P$ -value  $\leq 0.001$ ) and in function of

**Table 2.** Results of PERMANOVA analyses based on Jaccard distance matrices for each dataset

Dataset	Variable	$df$	SumsOfSqs	MeanSqs	FModel	$R^2$	Pr(>F)
Spring	Species	1	0.64	0.64	1.81	0.08	<b>0.001</b>
	Sex	1	0.33	0.33	0.94	0.04	0.596
	Main island district	1	0.86	0.86	2.42	0.11	<b>0.001</b>
	Islet/population	6	3.32	0.55	1.56	0.42	<b>0.001</b>
	Residuals	8	2.84	0.36	NA	0.36	NA
	Total	17	8.01	NA	NA	1.00	NA
Summer	Species	1	0.68	0.68	1.95	0.08	<b>0.004</b>
	Sex	1	0.37	0.37	1.06	0.04	0.372
	Main island district	3	1.63	0.54	1.56	0.18	<b>0.001</b>
	Islet/population	6	3.15	0.53	1.51	0.35	<b>0.001</b>
	Residuals	9	3.12	0.35	NA	0.35	NA
	Total	20	8.94	NA	NA	1	NA
Autumn*	Sex	1	0.26	0.26	0.85	0.09	0.772
	Main island district	1	0.55	0.55	1.76	0.19	<b>0.035</b>
	Islet/population	2	1.13	0.57	1.83	0.39	<b>0.007</b>
	Residuals	3	0.93	0.31	NA	0.32	NA
	Total	7	2.87	NA	NA	1	NA
Spring + Summer	Species	1	0.64	0.64	1.90	0.12	<b>0.001</b>
	Islet/population	2	1.45	0.73	2.15	0.26	<b>0.001</b>
	Season	1	0.53	0.53	1.57	0.10	<b>0.018</b>
	Population:Season	2	0.86	0.43	1.27	0.16	0.057
	Residuals	6	2.03	0.34	NA	0.37	NA
	Total	12	5.51	NA	NA	1	NA
Summer + Autumn	Islet/population	3	2.01	0.67	1.96	0.32	<b>0.001</b>
	Season	1	0.53	0.53	1.56	0.08	<b>0.028</b>
	Population:Season	3	1.09	0.36	1.06	0.17	0.318
	Residuals	8	2.74	0.34	NA	0.43	NA
	Total	15	6.37	NA	NA	1	NA

Significant  $P$ -values (< 0.05) are highlighted in bold.

\*The autumn dataset contained only *P. lilfordi* samples and therefore was not tested for the "species" variable.

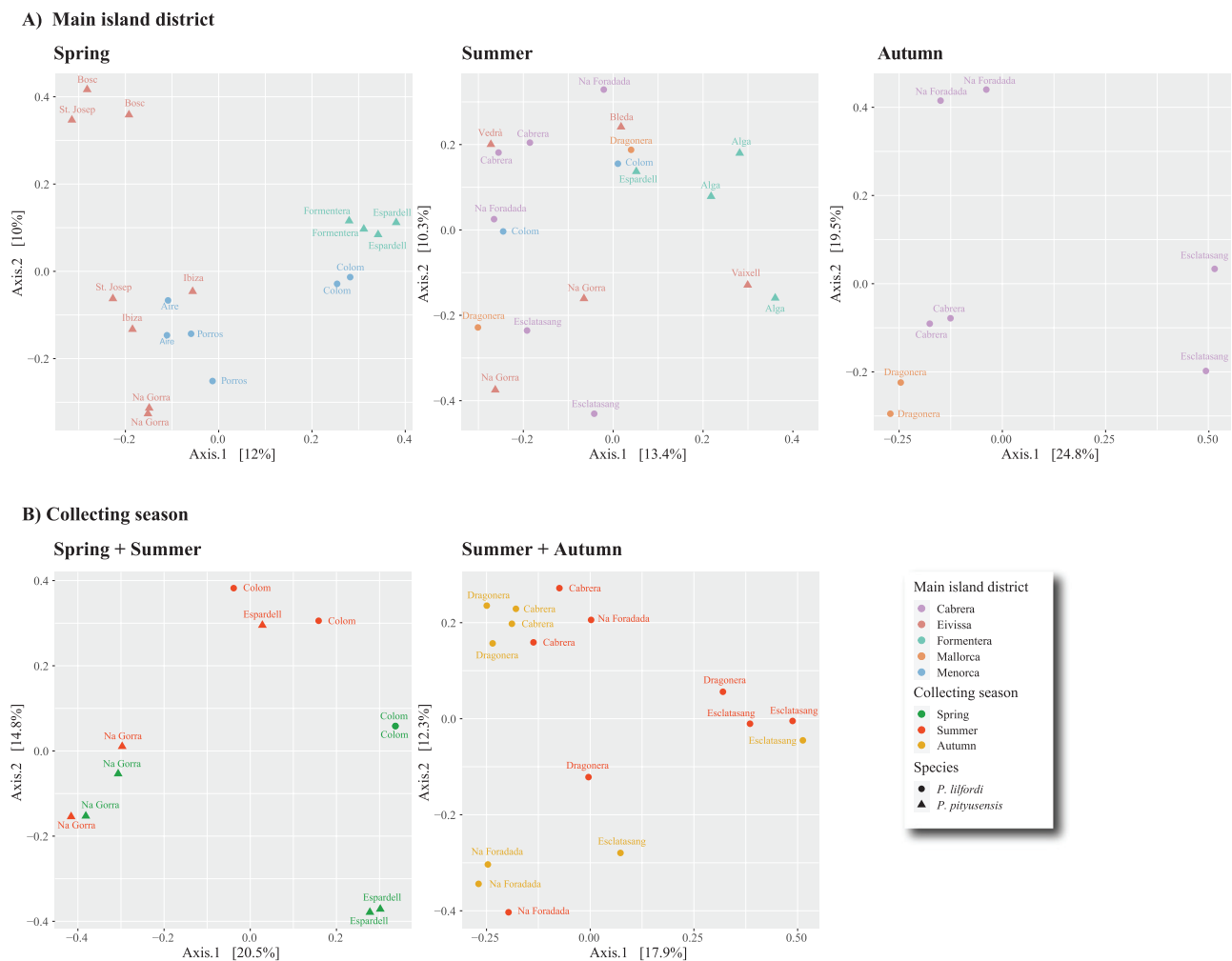
the collecting season variable ( $R^2$  range = 0.08–0.10,  $P$ -value  $\leq 0.028$ ) (Table 2). PCoA analyses based on Jaccard distance matrices were consistent with these findings (Figure 4).

Fisher's  $F$ -test, Student's two-sample  $t$ -test and Welch  $t$ -statistic to check for statistical differences in the number of diet OTUs per islet/population between lizard species retrieved significant values regarding the consumption of Julida in the Spring dataset (Welch  $t$ -statistic  $P$ -value = 0.025; Table 3).

## Discussion

The use of two different pairs of primers for each of the two main groups of organisms consumed by Balearic lizards (animals and plants) has allowed us to gather information on their trophic spectrum with a high level of taxonomic detail. Arthropods represent an important fraction of the diet of Balearic lizards (Pérez-Cembranos et al. 2016) and consistently the implementation of an arthropod-specific oligo pair has resulted in the recovery of a large number of OTUs compared to that reported by the COI universal primer pair. In addition, and despite the fact that the arthropod-specific primer pair targets a shorter COI region compared with that amplified by the universal mlCIIintF/dgHCO2198 pair (236 vs 313bp), it generated more precise taxonomic

identifications that, in most cases, reached the species level. Since both primer pairs target overlapping regions of the mitochondrial COI gene, differences in terms of identification accuracy could be attributed to the greater completeness of DNA databases regarding arthropod sequences compared to other animal groups. However, and despite the greater contribution of the arthropod-specific primer pair, our results show that both COI primer pairs complement each other. This is supported by the fact that the mlCIIintF/dgHCO2198 combination detected 4 orders, 14 families, 14 genera and up to 6 species of prey animals that went unnoticed for the arthropod-specific oligos. Regarding the plant fraction of the diet, the implementation of two molecular markers widely used in the field of plant DNA Barcoding has allowed us to expand our knowledge about the herbivorous habits of the Balearic *Podarcis* with a level of taxonomic precision that in most cases reached the genus and/or family ranks. The highest identification efficiency of the *psbA-trnH* chloroplast intergenic spacer reported here is consistent with previous studies (Pang et al. 2012). In addition, *psbA-trnH* also reported a higher number of OTUs at all taxonomic levels compared with the *rbcL* marker, a fact that could be explained by the existence of priming sites within highly conserved flanking coding sequences and a highly variable non-coding region



**Figure 4.** Principal coordinate analyses based on the Jaccard distance matrices from the five analyzed datasets (see main text for details); (A) highlighting the main island district effect (dot colors) and lizard species (dot shapes); (B) highlighting the effect of season (dot colors) and lizard species (dot shapes) (See online version for color figure).



**Table 3.** Results of the Fisher's *F*-test to check for statistical differences in the number of diet OTUs per islet/population between lizard species for each of the orders that constitute at least 5% of the diet of either *P. lilfordi* or *P. pityusensis*

	Order	Spring			Summer		
		var.test	Student's <i>t</i> -test	Welch <i>t</i> -statistic	var.test	Student's <i>t</i> -test	Welch <i>t</i> -statistic
Animals	Coleoptera	–	–	–	0.111	0.724	–
	Diptera	0.463	0.366	–	0.167	0.468	–
	Geophilomorpha	–	–	–	<b>0.000</b>	–	0.177
	Hemiptera	0.463	0.644	–	0.259	0.880	–
	Hymenoptera	0.821	0.351	–	0.259	0.057	–
	Isopoda	0.640	0.286	–	0.232	0.657	–
	Julida	<b>0.000</b>	–	<b>0.025</b>	<b>0.000</b>	–	0.070
	Lepidoptera	0.732	0.252	–	–	–	–
	Sarcoptiformes	0.726	0.407	–	–	–	–
	Stylommatophora	0.615	0.685	–	–	–	–
	Trombidiformes	0.821	0.351	–	–	–	–
Plants	Asterales	0.821	0.351	–	0.532	0.166	–
	Caryophyllales	0.1283	0.5527	–	0.432	0.824	–
	Pinales	–	–	–	<b>0.000</b>	–	0.175
	Pottiales	–	–	–	<b>0.000</b>	–	0.374

Significant *P*-values (< 0.05) are highlighted in bold.

(Kress and Erickson 2007). In any case, and even though the relative performance of the *rbcL* marker is lower, our study has benefited from its inclusion as evidenced by the contribution of 1 plant class, 2 orders, 3 families and a genus to the final list of trophic resources consumed by the Balearic lizards.

The high-throughput sequencing metabarcoding approach implemented here supports the omnivorous and highly variable character of the diet of the Balearic *Podarcis* lizards. Our results show a trophic spectrum composed mainly of a diverse array of arthropod and mollusk animal prey, and vegetal tissues of terrestrial plants including mosses, monocots, and dicots. According to the weighted proportion of occurrence of diet items provided by our DNA-based approach, OTUs from orders Coleoptera, Diptera, Hemiptera, Hymenoptera, Isopoda, Lepidoptera and Trombidiformes, as well as for plant tissues from orders Caryophyllales and Pottiales were consistently preyed upon by both *Podarcis* species across all sampled seasons. Although many of these main taxonomic groups had already been reported previously as part of the diet of the Balearic *Podarcis* based on macroscopic methods (Salvador 1986b; Pérez-Mellado 1989; Pérez-Mellado and Corti 1993; Traveset 1995; Pérez-Cembranos et al. 2016; Santamaría et al. 2020), the most important contribution of our study lies in the higher level of resolution of the taxonomic identifications and in the detection of new trophic records.

The resolution capacity of our DNA-based approach is clearly illustrated in the case of the ants, a trophic resource known to be particularly abundant in the diet of *P. lilfordi* (Pérez-Cembranos et al. 2016) and that we could ascribe to genera *Crematogaster* (including the species *C. scutellaris*), *Lassi* (*L. lasioides*), *Messor* (*M. bowieri*), *Pheidole*, *Tapinoma*, and *Tetramorium* (*T. semilaeve*). Similarly, our DNA metabarcoding results also expands our knowledge on beetle consumption—the most important prey group in terms of ingested biomass (Pérez-Cembranos et al. 2016)—to families

*Anobiidae* (genus *Gastrallus*), Scarabaeidae (*Polyphylla*), and Tenebrionidae including species from genera *Asida* and *Blaps*. These expansion of knowledge about the taxonomic details of the diet of Balearic lizards extends to the rest of the animal prey and plant orders detected, and it acquires special relevance in cases such as Lepidoptera, for which previous reports based on macroscopic methods have only detected poorly identified remains of larval structures (Santamaría et al. 2020) and our DNA-based analysis could ascribe to eight butterfly families including 11 genera and eight species. Such level of taxonomic resolution is highly dependent on the information available in DNA sequence databases, which currently prevent the complete taxonomic identification of many of the sequences (Wangensteen et al. 2018) (Supplementary Table S1). Thus, the identification rate of the diet DNA reads here reported will further increase as the taxonomic gaps in the databases are filled. A complementary approach in this regard would be the building of a local database of reference DNA sequences from the fauna and flora inhabiting the islets populated by the Balearic lizards. Unraveling the taxonomic details of trophic associations of *P. lilfordi* and *P. pityusensis* would not only provide valuable information about their biology but would also enable the compilation of high-quality ecological interaction networks necessary to understand ecosystem functionality (Cuff et al. 2022) and to design effective conservation strategies (Harvey et al. 2017).

In addition to a higher taxonomic resolution capacity compared to classical diet analysis, this first implementation of the metabarcoding approach in the Balearic lizards has reported new trophic records that substantially expand our knowledge about their omnivorous ecology. Our findings demonstrate the ability of the Balearic lizards to adapt to the scarce resources available in their isolated habitats as evidenced by the consumption of marine subsidies, vertebrate carrion, and mosses among other prey items from the orders Decapoda, Mesostigmata, Pelecaniformes, Psocoptera, Rhabditida, Sarcoptiformes, Scolopendromorpha, Scutigermorpha,

Geophilomorpha, Trombidiformes, and Thysanoptera. Prey decapods are represented in our results by the marbled rock crab *Pachygrapsus marmoratus* which is very abundant in the coastal environments of the Balearic archipelago (Crocetta et al. 2011) and the Argentine red shrimp *Pleoticus muelleri* which is a very common commercial shrimp in local markets whose remains could have reached the population where it has been detected (Na Gorra) through seabirds such as the Yellow-legged Gull, a very abundant seagull species in the archipelago known to feed on open-air dumps (Mas et al. 2015). The feeding on Phalacrocoracidae reported from *P. pityusensis* Alga and Na Gorra populations can be related to *Phalacrocorax* since it is the only genus of this bird family inhabiting the Balearic territory (Vicens 2012) and provides another interesting record of carrion consumption that previous studies could only report based on unidentified remains of feathers and/or hairs (Salvador 1986b). Our study also contributes the first record of feeding on psocopterans for both *P. lilfordi* and *P. pityusensis*, a prey group known to be consumed by other *Podarcis* species (Bombi et al. 2005). Also interesting is the case of the mites (Sarcoptiformes) detected in our analyses and represented by families and genera such as *Bonomoia*, *Scutovertex* and *Tyrophagus* typically associated with insects, vertebrate nests, plants, mosses, lichens, or fungi (Fan and Zhang 2007; Schäffer et al. 2010; Wirth 2016), some of them adapted to live in sun exposed rocks in salty environments (*Scutovertex*; Schäffer et al. 2010). This finding is consistent with previous reports of Sarcoptiformes DNA from fecal samples of lacertid lizards (Pereira et al. 2019). On the other hand, the consumption of undetermined myriapods by Balearic *Podarcis* demonstrated by macroscopic analyses of fecal contents (Salvador 1986b; Pérez-Mellado and Corti 1993) can be updated with a higher level of taxonomic precision based on our trophic inferences on Geophilomorpha, Scolopendromorpha (genus *Cryptops*), Scutigermorpha (*Scutigera coleoptrata*) and Julida (*Ommatoiulus*). Thrip consumption (Thysanoptera) by *P. pityusensis* is also a new finding derived from our results that is consistent with previous reports of this insect group in the diet of other *Podarcis* species from the Iberian Peninsula (Escarré and Vericad 1981). New trophic records from order Trombidiformes include OTUs from families Eriophyidae (gall mites) and Tarsonemidae (thread-footed mites), both plant parasites that due to the combination of their small size and the degrading effect of the digestive processes could have gone unnoticed by classical macroscopic approaches. Our study also contributes new reports of trophic associations regarding plant groups that represent an important portion of the diversity of the diet of both *P. lilfordi* and *P. pityusensis*. They include the feeding on OTUs from two plant orders not previously reported (Brassicales and Malvales) as well as new associations with genera from orders already known to be consumed: *Sonchus* (Asterales), *Beta* and *Limonium* (Caryophyllales), *Daucus* (Apiales), *Asparagus* (Asparagales), and *Erodium* (Geraniales). The feeding on mosses by the Balearic *Podarcis* lizards is also an unprecedented finding. These new reports represent a substantial advance in understanding the ecological role of both lizard species, whose trophic spectra are probably much broader if we consider that DNA-based inferences only provide information regarding feeding events that occurred a few hours before fecal sampling (Thuvo et al. 2019). In this regard, an experimental design based on frequent long-term sampling would provide a more complete view of the highly diverse diets of these *Podarcis* species.

Accidental and/or secondary ingestion can be error sources when investigating omnivorous diets (Terceel et al. 2021). This could be the case of the inferred trophic associations with gymnosperms since chloroplast genomes are paternally inherited in most conifer taxa including those from orders Cupressales and Pinales reported by our study (Adams 2019). Therefore, the accumulation of pollen grains from these plant groups on the food items of the lizards could be enough for the specific PCR primers of the chloroplast genome that we have used in this study (molecular markers *psbA-trnH* and *rbcl*) to amplify them. Similarly, the finding of DNA remains from Rhabditida in the analyzed *Podarcis* fecal samples could not necessarily be indicative of a trophic relationship since this nematode group includes zooparasitic helminths of lizards (Moravec 2010), and therefore further research would be needed to clarify their presence in the analyzed scats.

PERMANOVA analyses based both on samples from the same collecting season (i.e., spring and summer datasets) and on samples from populations with matching collecting seasons (i.e., Spring + Summer and Summer + Autumn datasets) consistently yielded significant differences in terms of diet composition between *P. lilfordi* and *P. pityusensis*. In addition, our niche overlap analyses significantly reported a moderate resemblance in resource usage between summer samples from both lizard species (Pianka's index 0.526), meaning that an important fraction of the diet of each species is exclusive. As evidenced by our results, such differences are not related with the size of the islands inhabited by both lizard species and could rather be explained by their evolutionary separation ca. 5.3 Ma. (Brown et al. 2008) (ca. 3.5 Ma. *sensu* Salvi et al. 2021) and their a non-overlapping segregated distribution in the Balearic archipelago each inhabiting well-differentiated biogeographic districts (Rivas-Martínez et al. 2017). Indeed, there exist differences in terms of flora composition between the eastern islets where *P. lilfordi* inhabits (Mallorca, Menorca and Cabrera district) and those located in the western part of the archipelago populated by *P. pityusensis* (Ibiza and Formentera district) (Rita and Bibiloni 2013). Such inter-specific dietary differences agree with previous reports based on macroscopic analyses of stomach and fecal pellet contents (Pérez-Mellado and Corti 1993). Niche breadth of *P. lilfordi* showed similar Levin's index values across seasons while disparate values were observed for *P. pityusensis* corresponding to a high trophic spectrum in spring and a reduced niche width in summer. These observations are in line with the analyses of seasonal effect on the diet of the Balearic *Podarcis* conducted by Pérez-Mellado and Corti (1993) that concluded that dietary diversity is lower in summer than in spring. This could be related with the warm and dry summer conditions that characterize the Mediterranean climate in contrast with the favorable spring conditions for both flora and fauna. The seasonal dietary variation is also supported by our PERMANOVA analyses based on samples from populations with matching collecting seasons.

In addition to seasonality, the geographic distribution of lizard populations also appears to be a determining factor in explaining dietary variation. Main Balearic island district and islet/population variables were found to significantly influence the observed pattern of resources consumption, which could be related to the similarity in terms of environmental conditions and available trophic resources in nearby locations. This view is supported by our niche overlap analyses based on

Pianka's index where significantly similar resource utilization spectra were derived from pairwise diet comparisons among populations from geographically close islets in the surroundings of Ibiza and Formentera (Alga, Bleda, Espardell, Vaixell and Vedrà).

In conclusion, the metabarcoding strategy used here and based on non-invasive sampling has made it possible to characterize with a high degree of taxonomic detail the highly variable diets of the endemic *Podarcis* lizards of the Balearic archipelago, including new trophic records. The use of two different molecular markers for each of the main groups of organisms that make up the diet of lizards (animals and plants) has proven to be an effective strategy. The diet compositions of *P. lilfordi* and *P. pityusensis* differ inter- and intra-specifically both at the seasonal and the geographical level and are marked by the consumption of a high diversity of arthropods, mollusks, and plants, that also include feeding on carrion and on marine subsidies. Our molecular results complement those from previous studies based on macroscopic analyses of diet remains and provide a comprehensive view of the diversity of ecological interactions that characterize the populations of these threatened endemic lizards in their unique isolated ecosystems.

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## Conflict of Interest Statement

The authors declare that they have no conflict of interest.

## Authors' Contributions

IA, JAJ-R, CR and VPM designed the research. IA, VPM and AP-C collected the fecal samples. IA and JAJ-R performed the molecular work, performed bioinformatics data analyses and generated the figures. CR, VPM, JAC, AP and JAJ-R obtained the funding. JAJ-R, IA and CR drafted a first version of the manuscript with contributions of other authors to the final version.

## Data accessibility

Raw sequences are available in the Sequence Read Archive (SRA) database at NCBI under BioProject ID PRJNA703933.

## Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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