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Metabarcoding assessment of the diet of an introduced continental lizard to an oceanic island reveals dietary niche conservatism

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Invasive species can have devastating effects when introduced into remote island ecosystems, and a fundamental aspect of this concerns the diet of these exotic taxa. Here, we employed a DNA metabarcoding approach to determine the diet of the lizard *Agama picticauda* on Réunion Island, where it was introduced in 1995. Two separate markers were used to identify dietary components: COI for animals and trnL for plants. The arthropod aspect was notably conservative, with the agama continuing to predominantly consume ants, as they do in their native range. A variety of other invertebrates were also preyed upon, the vast majority being introduced species. For plants, again a wide variety was detected, and while most could not be identified fully, it seems that agamas are deliberately consuming many species, rather than accidentally ingesting them along with targeted invertebrates. Agamas may play a role in seed dispersal of invasive plant species. We also detected some nematode groups, although with limited comparative sequences, these could not be identified to the species level. Several invertebrate records appear to be new for Réunion Island, highlighting how reptiles can be considered as excellent biodiversity samplers, with barcoding diet studies providing novel data on poorly known invertebrate groups. The minimal identification of endemic prey items may reflect the fact that agamas are still predominantly occupying anthropogenically disturbed parts of the island. Our study therefore provides baseline data that can be used to determine the impact of this introduced lizard as it spreads through the ecosystem.

The main drivers of biodiversity loss include habitat fragmentation, climate change and invasive species. Furthermore, it has been well-documented that the majority of vertebrate extinctions in recent times have occurred on islands and that the bulk of these were associated with the establishment of exotic species[1–3]. Island reptiles are particularly threatened by introduced competitors, especially on islands that were previously predator-free[4]. Because of this, there have been extensive studies on the impact of invasive species such as rodents or cats on island endemic reptiles (e.g.[5,6]). Invasive reptiles have also been associated with extinctions, for example, the introduced gecko *Hemidactylus frenatus* was linked to the decline and extinction of endemic night gecko *Nactus* populations on the Mascarene Islands[7]. However, there have been fewer studies on the impact of invasive introduced reptiles on consumed taxa, because this trophic interaction involving many different potential prey items is harder to determine, and disentangling the mechanism through which introduced species impact the ecosystem remains a key challenge in ecological research[8]. The development of metabarcoding approaches, in which dietary items are identified using next-generation genetic sequencing tools, offers a useful new technique to address this issue, particularly since prey items can be identified to much higher taxonomic levels than typically determined using microscopy assessments of stomach contents or pellets (e.g.[5,9]).

The Peters's rock agama *Agama picticauda* is a common and widespread lizard in sub-Saharan West Africa (Fig. 1), where it is often found in urban environments[10,11]. It is a highly-successful invasive species[12]; for example,

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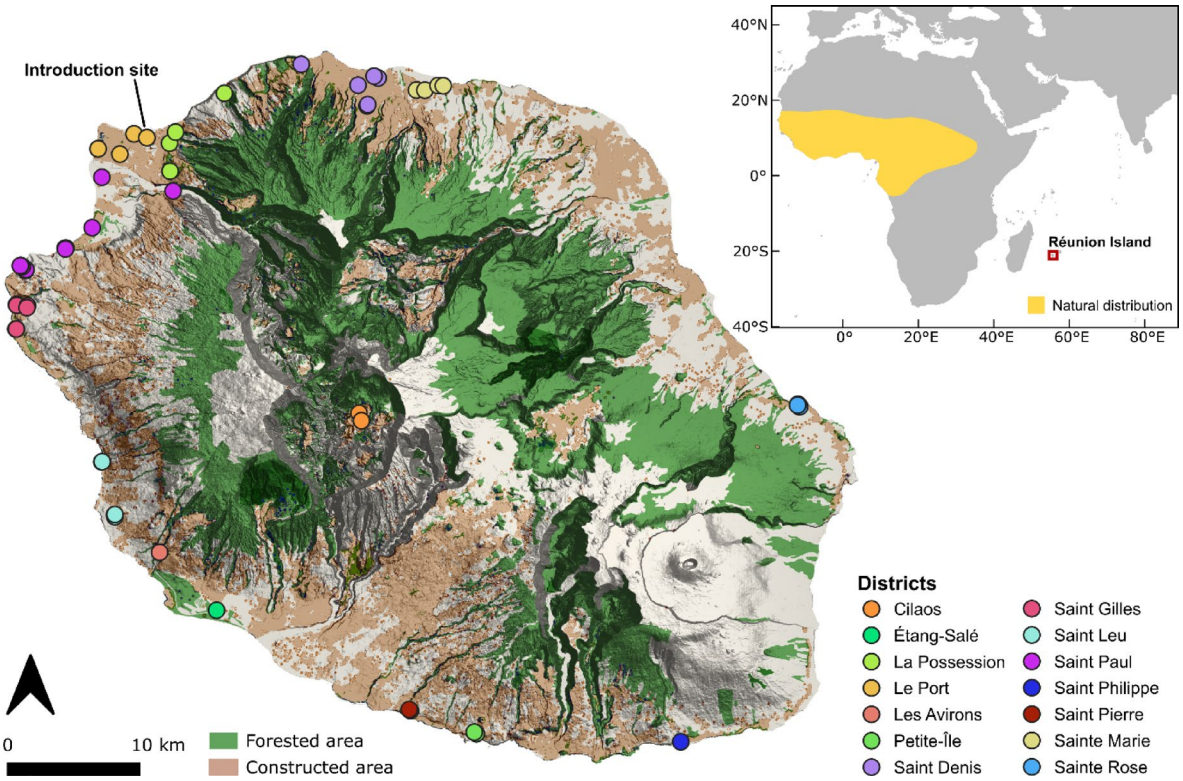


Fig. 1. Map showing the sampling localities of *Agama picticauda* from across Réunion Island.

in Florida, USA, it is one of the most rapidly spreading non-native herpetofauna[13]. In the Caribbean Lesser Antilles, *A. picticauda* has the potential to have a major impact on numerous native species if it spreads further and establishes populations on new islands[14]. Recent molecular analyses have confirmed that the populations on Grande Comore and Réunion Island were independently introduced to these islands[15]. On Réunion Island, the first individuals were reported near the main maritime port at Le Port approximately 30 years ago[16], and are now spread across coastal areas and, more recently, into higher-elevation central region[15]. In their native range, *A. picticauda* prey predominantly on insects, with Hymenoptera (Formicidae) and Coleoptera being the most frequently consumed prey orders[17]. Morphological identification of stomach contents identified prey items from 14 orders, indicating a relatively wide prey spectrum[17]. *Agama picticauda* may also consume plant matter, small vertebrates, and even scavenge opportunistically on anthropogenic processed foods such as bread[11].

The Mascarenes, consisting of the volcanic islands of Réunion, Mauritius, Rodrigues, as well as various small coralline islands, form part of the world's top biodiversity hotspots[18]. Réunion Island is the largest island (2512 km^2), with dated lavas of around 2.1 million years. Although severely fragmented, about 25% of the estimated original extent of habitats on Réunion Island remain in a relatively good state. Like other similar island groups such as the Hawaiian Islands, the Mascarene biota includes notable levels of endemism, including around 75% of native flowering plants and 90% of nonmarine molluscs[18]. Of the terrestrial arthropods on Réunion Island, 31% are endemic to the island, and 40% endemic to the Mascarenes, although these are rough estimations given that around 60% of the arthropod fauna is predicted to remain unnamed[19].

Given the rapid spread of *A. picticauda* across Réunion Island and the high level of endemic and endangered potential prey items, an assessment of the trophic niche of this introduced agamid is clearly needed as part of the recently established "invasive species strategy and action plan", which aims to mitigate its spread and associated risks[20]. Here, we used a metabarcoding approach to assess the diet of 66 adult individuals, using stomach contents collected from populations around the island, including the higher elevation central region (Fig. 1). We aimed to collect baseline data regarding prey items, and to determine if known endemic or endangered species were being preyed upon. The data can also be compared with the known diet from its native range to assess levels of trophic niche conservatism. At the same time, dietary DNA or dDNA (*sensu*[21]) can often give notable new information about prey items themselves, helping to establish reliable barcoding databases of species presence. Furthermore, sequences of endoparasites such as nematodes can sometimes be identified in stomachs using this genetic approach, furnishing additional information on these poorly known species, potentially introduced along with their lizard host.

Results

The COI and trnL libraries generated ca. 6.4 million raw sequence reads, which were reduced to 171,419 reads during the bioinformatic processing and to 558 total OTUs. Non-target COI amplification from different sources was observed both in samples, extractions, and PCR negative controls, representing approximately 50% of the

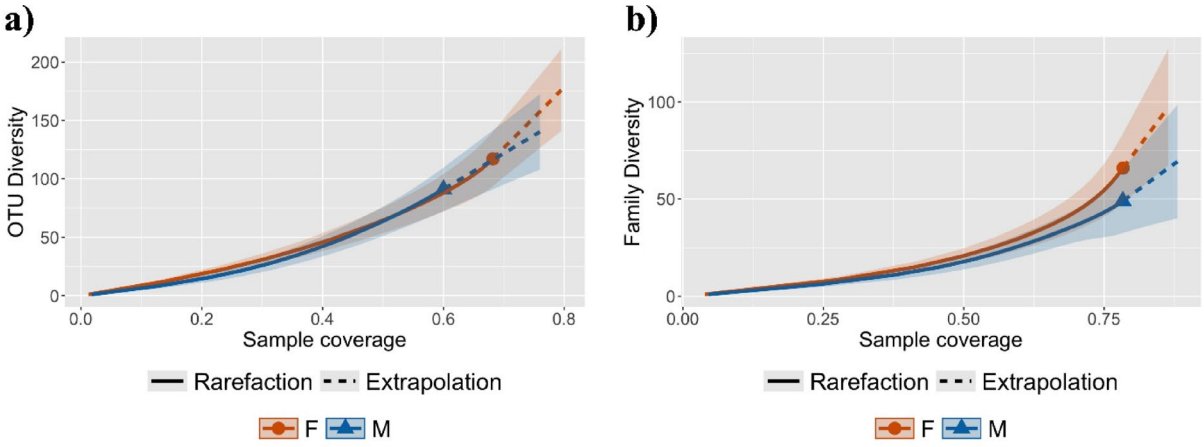


Fig. 2. Rarefaction curves for males and females at the OTU (**a**) and family (**b**) levels, showing the observed (full line) and estimated (dashed line) richness, until double the reference sample size, and respective 84% confidence interval by sample coverage.

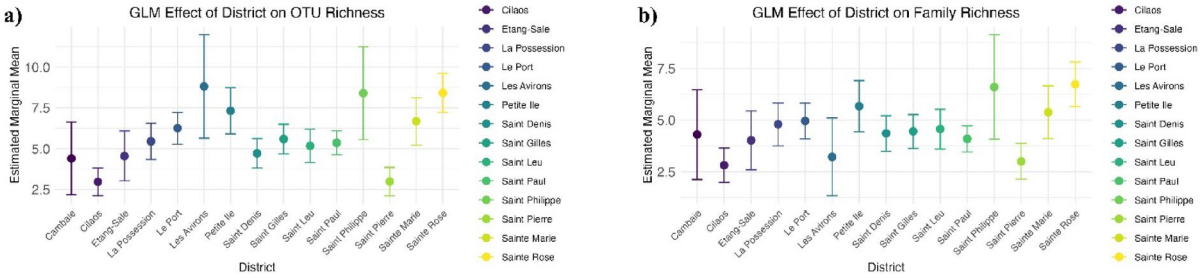


Fig. 3. Graphical representation of the General Linear Models results denoting the relationships between populations with OTU richness (**a**), and family richness (**b**).

total reads. Almost all of these corresponded to the host, *Agama* (48.19% of the total reads), while Nematoda represented around 1.25% of the total reads, and Fungi 0.04%. After negative controls, singletons, replicates, and taxa filtering, the lizards' final diet using the COI primers consisted in 20,655 reads and 68 animal OTUs from 3 phyla, Annelida, Chordata and Arthropoda. Prey items were considered to belong to 38 families of arthropods, 2 families of annelids, and 1 family of chordates (geckos) (Supp. Material Table S2). The most frequent OTUs were generally ants (Formicidae), in particular *Paratrechina longicornis* (49.15% FO), *Brachymyrmex cordemoyi* (11.86%), *Solenopsis geminate* (8.47%) and *Pheidole megacephala* (6.78%). The most frequent prey OTUs after ants were the honey bee (Apidae) *Apis mellifera* (18.64%) and the beetle (Coccinellidae) *Exochomus laeviusculus* (15.25%). No other prey items had frequencies over 6%. As well as the prey items, 4 OTUs corresponded to nematodes from the orders Rhabditida and Spirurida. Regarding the chloroplast primers, a total of 52,149 reads were obtained and 97 OTUs were detected (7 up to the species level), corresponding to 44 families (Supp. Material Table S3).

Growth trajectories were similar for males and females, but males grew both bigger and heavier (Suppl. Material Fig. S1). There were significant differences in SVL between districts ($F = 2.35$, $p = 0.01$) and sexes ($F = 40.21$, $p < 0.001$), but weight differences were only significant between the sexes ($F = 32.80$, $p < 0.001$) and not between districts ($F = 1.72$, $p = 0.08$). Female agamas had higher dietary richness than males at both the prey OTU and family level, but the differences were not significant. Analysis of rarefaction and extrapolation curves (Fig. 2) indicate no significant differences between males and females, with overlapping dietary niches. According to the GLM analysis, there was significant differences in OTU richness between districts (District: df = 14, Deviance = 16.42, Residual Deviance = 36.66, p-value = 0.29; Sex: df = 1, Deviance = 0.88, Residual Deviance = 35.78, p-value = 0.35; logSVL: df = 1, Deviance = 0.01, Residual Deviance = 35.78, p-value = 0.94), but none of the pairwise comparisons were significant (Fig. 3). At the family level there were no significant results in richness ((District: df = 14, Deviance = 29.13, Residual Deviance = 54.82, p-value = 0.01; Sex: df = 1, Deviance = 1.88, Residual Deviance = 52.95, p-value = 0.17; logSVL: df = 1, Deviance = 0.21, Residual Deviance = 52.73, p-value = 0.64). Regarding the analysis of diet composition, there were significant differences only between districts at the OTU level, but not at higher taxonomic levels (Table 1). These significant results are not related to the dispersal of the data ($F = 1.54$, $p = 0.15$).

	df	Sum of squares	*R* squared	F model	*p*-value
OTU					
District	14	7.32	0.24	1.18	**0.00**
Sex	1	0.50	0.02	1.14	0.17
logSVL	1	0.42	0.01	0.95	0.57
Residual	51	22.51	0.73	NA	NA
Family					
District	14	5.93	0.22	1.07	0.19
Sex	1	0.36	0.01	0.91	0.57
logSVL	1	0.28	0.01	0.72	0.86
Residual	51	20.27	0.75	NA	NA

Table 1. Results from PerMANOVA on the effect of District, Sex and logSVL on OTU and family diet composition. Df stands for degrees of freedom. Significant *p*-values are highlighted in bold.

Discussion

An assessment of the diet of *A. picticauda* along a transect of over 800 km within its native range along the west African coast showed that all populations were mainly insectivorous and that food niche overlap was high between all populations[22]. The most frequently identified prey item was ants (Formicoidea), with frequencies ranging from 20 to 53%. Coleoptera, Lepidoptera, Araneidae and Vespoidea were also found in all populations sampled. Dietary niche overlap decreased with increases in the difference of mean annual rainfall between sites, but there was no effect of geographic distance. Similarly, in a population from Nigeria, the most frequent prey items identified were black ants, Blattodea (Isoptera, termites), yellow ants, bees and wasps, with melon seeds also more frequent than some arthropod orders[23].

In this study, the diet of the introduced *A. picticauda* on Réunion Island shows notable similarities with the populations from the native range, indicating considerable trophic niche conservatism. Again, the most frequently identified prey items were ants. Eleven OTUs corresponding to 8 genera within Formicidae were recorded (Suppl. Material Table S2), of which *Paratrechina longicornis* (49.15%), *Brachymyrmex cordemoyi* (11.86%), *Solenopsis geminate* (8.47%) and *Pheidole megacephala* (6.78%). were the most frequently recovered, representing 76.26% of all prey OTUs identified with these primers. Coleoptera, Lepidoptera, Araneidae and Vespoidea were also all found at high frequency, with Coleoptera also representing an important part of the species' diet in West Africa[10,17]. Other less widely identified prey items from the native range, such as Isopoda, Diptera, and Blattodea, were also identified as prey items in Réunion Island. Differences included, for example, that in West Africa, scorpions were occasionally consumed (up to 1.8% frequency in 3 of 8 sampled populations), while these were not detected in Réunion Island, although this is unsurprising since only two introduced species are known from this island[19].

An acknowledged advantage of the use of metabarcoding in lizard diet studies is the ability to determine prey items to a much higher level than typically obtained using microscopy, and this precision can sometimes uncover previously unknown aspects of the trophic niche (e.g.[24]). Previous assessments of the invertebrate prey items of *A. picticauda* determined using microscopy had identified prey predominantly only to the order or family level[22,23]. A more recent extensive assessment of a population of *A. picticauda* from urban and rural populations in Ghana identified 14 orders and 47 families[17]. In this study, 12 arthropod orders and 41 families were identified, again demonstrating the wide breadth of the dietary niche of *A. picticauda* – while ants and a few other groups make up the bulk of the diet, an extremely wide diversity of arthropods and other small invertebrates are consumed.

The precision of prey identification means that metabarcoding studies of lizard pellets can also provide new data regarding the prey items themselves, especially since distribution data for invertebrates is often imprecise. For example, in an assessment of the diet of the wall lizard *Podarcis lusitanicus*in Northern Portugal, Simões et al.,[25], reported several invertebrate prey species that were apparently new records for this country. Fortunately, the terrestrial arthropod fauna of Réunion Island has been relatively well studied. A recent review identified 3,369 species, of which 31% were endemic to Réunion Island[19]. Endemism rates, however, vary widely between groups, with for example, 21% of spiders (Araneae) endemic, but only one ant (Formicidae) species out of 48 being endemic. Furthermore, it has been estimated that 62% of all the terrestrial arthropod fauna on Réunion Island remains to be named[19]. Identified prey items may therefore be useful in highlighting undescribed diversity, as well as allowing identification of endangered or endemic species within the trophic niche, as opposed to introduced and widespread species. Identified prey items may also represent first reports of introduced species that can then be added to update the faunistic catalogues of the island. For example, the spider *Oecobius putus* is not in the most recent, exhaustive checklist for the island[26] or in global databases such as GBIF (http://www.gbif.org/) and may represent a first record for Réunion Island. Likewise, the earthworms *Travoscolides chengannures* and *Pontoscolex corethrurus* are neither in the GBIF database nor dedicated earthworm databases (http://taxo.drilobase.org/) for Réunion Island. The same occurs with the millipede *Leptogoniulus sorornus* and the beetle *Myrmechixenus vaporariorum*. These potentially new records for Réunion Island are species that are poorly studied and likely introduced. Regarding *Oecobius* on Réunion Island, Cazanove[26], states that "some species may have gone unnoticed due to a lack of attention and specific sampling". Similarly, *P. corethrurus* is widely introduced, and already reported as such to Mauritius, the Comoros, and the Seychelles[27].

The characterisation of ants in the diet of *A. picticauda* gives an example of the advantages of the barcoding technique over classic microscopy studies. All previous assessments had noted the high proportion of prey items of the family Formicidae. However, our study distinguished 11 OTUs, or presumed species, 10 of which were identified to the species level, within 8 genera. This means that of the 48 ant species known from Réunion Island, 22% were apparently part of the diet of *A. picticauda*. All of the identified ants were widespread, introduced species – the single known endemic ant species was not found. This is relatively good news regarding the impact of *A. picticauda* on native arthropods, as it appears to maintain a preference for ants, the vast majority of which are introduced on Réunion Island. Previous studies have suggested that *A. picticauda* may consume a high frequency of ants as they are among the most frequently encountered ground-dwelling insects[17]. However, our finding of such a wide diversity of ant species seems to rather indicate that ants are preferentially preyed upon, which may also help explain the conservatism of the trophic niche in this introduced population.

Given that around one-third of the terrestrial arthropods on Réunion Island are endemic, it might have been expected to find a similar proportion within the identified prey items. Instead, only one endemic species, the Hemiptera *Deraeocoris howanus*, was recognised, out of 20 identified to the species level. Although *A. picticauda* predominantly consumes introduced species, this does not mean that the lizard has no considerable impact on native terrestrial arthropods. Introduction of an exotic lizard has been shown to significantly alter ant community structure, reducing the abundance of some species and therefore having indirect effects on other species[28]. Furthermore, the broad dietary niche of *A. picticauda* means that many species are consumed, and the unidentified prey items may correspond to additional endemic species. A more complete database of sequences from island endemics would be needed to further assess this.

Another aspect of the threat caused by introduced species is that they can bring parasites with them, which may have severe impacts if they are transmitted to local hosts. An interesting bycatch of the COI primers employed in this study, and widely used for assessing the diet of reptiles, is that they can also amplify nematodes. In this study of *A. picticauda* introduced to Réunion Island, four distinct OTUs corresponded to nematodes. A previous morphological assessment of gastrointestinal helminths from *A. picticauda* within its native range in Ghana identified four helminth species, *Ascaris* spp., *Enterobius* spp., *Pharyngodon* spp. and *Oxyurid* spp.[17]. Unfortunately, none of the nematode sequences from this study had close matches on GenBank (83–93% matches), so we cannot ascertain with any degree of certainty which species they may correspond to. At the same time, morphological assessments of nematodes in other introduced populations of lizards have identified species typical of the local herpetofauna, indicating that the exotic species had acquired their helminth assemblage from the local helminth pool, rather than possessing species from the parasite fauna of the original population[29,30]. Although we cannot therefore currently identify these nematodes, as more sequence data becomes available for helminths from lizards, in the future, these sequences may be useful for identifying these species.

An additional potential threat from the introduced *A. picticauda* would be direct predation on endemic lizards, since agamas are known to occasionally prey on small vertebrates, and there are also records of cannibalism[23,31]. Cannibalism cannot be assessed with the metabarcoding approach used, as sequences corresponding to *A. picticauda* were automatically discarded as presumed belonging to the host individual. No sequences corresponding to *Phelusma* day geckos were identified, and although sampling locations do not overlap with the distribution of native *Phelsuma* spp.[32,33], they do overlap heavily with introduced *Phelsuma* spp.[34,12]. The introduced gecko *Hemidactylus frenatus* was identified at a low frequency (1.47%).

Agama picticauda populations typically exhibit sexual size dimorphism[14,15], with both sexes showing a similar SVL/weight trajectory, but with males being both larger and heavier (Suppl. Mat. Fig. S1). Given this, it might be presumed that the dietary niche of males would be larger than that of females, as their larger size would enable them to consume some prey items that would be too big for females to consume. Interestingly, however, the females had higher diet richness when assessed at both the OTU and family level, although the difference was not significant. In other lizards with sexual dimorphism, males have been shown to select larger prey items, corresponding to their larger head size and bite force[35]. On the other hand, in dDNA assessments, the size of prey consumed remains unknown – females and males could be preying on the same species, but selecting specimens of different sizes, for example. Rarefaction and extrapolation curves (Fig. 2) indicate that males and females have considerably overlapping dietary niches, with no significant differences observed.

Previous studies of diet variation along transects of *A. picticauda* populations have indicated that, while the preference for certain prey items such as ants and beetles remains constant, variation between populations does occur[22]. This is expected, as the terrestrial arthropod community will vary with geological factors such as rainfall, and also anthropogenic influences such as levels of urban development[17,22]. When assessing differences in dietary consumption between populations, significant differences were found in OTU richness between districts, but in pairwise comparisons, none are significant, probably because of the low sample numbers in some regions (Fig. 3). Furthermore, no significant differences were found between populations when assessing family-level richness. While *A. picticauda* is spreading around the island, they are still heavily associated with anthropogenically disturbed areas[20]. This may also explain why so much of the diet consists of introduced species, similarly associated with urbanised areas. The honey bee *Apis mellifera* (18.64%) is the second most frequent arthropod OTU in the diet of *A. picticauda* and, while introduced, represents a species of significant economic importance. Long-term monitoring will be needed to see if this dietary aspect changes with the continuing spread of agamas into more natural areas.

Our assessment of plant material in the stomach contents identified 97 OTUs from 44 families, with seven identified to the species level. Although the most common OTUs could not be fully identified (Unidentified Solanaceae 25%, Unidentified Fabaceae 22%), the third most frequent OTU was *Desmondium scorpiurus*(13%), a perennial tropical legume widely used to feed livestock. Rabiu[23], identified seeds in 37% of pellets within the native range of *A. picticauda*, but the most common were melon seeds, available through anthropogenic discards. In this study, various potential fruits were also identified, including *Ficus* sp. (figs), *Rubus* sp. (blackberries) and

Musa sp. (bananas). Combined with the high diversity of plant species detected, and the high nutritional value of many of them, it seems likely that *A. picticauda* are specifically targeting plants for consumption, rather than accidentally ingesting them while aiming at invertebrates. None of the plant species identified were endemic to Réunion Island. However, some may be seed dispersed by this lizard. For example, one of the species identified was the highly invasive Brazilian pepper tree, *Schinus terebinthifolius*. It is known that recruitment of this species is dependent upon frugivores –in Australia, seed germination is minimal without pulp removal, a task that is accomplished by a native bird species[36]. Previous studies of other introduced reptiles, including the green iguana in Puerto Rico[37] and the panther chameleon in Réunion Island[38] have found they also consume seeds of *S. terebinthifolius*. Agama may therefore play a role in the maintenance and spread of this invasive tree species, highlighting the complex interplay between introduced species in these small island communities.

Despite the clear advantages that metabarcoding approaches for dietary assessments have over microscopy appraisals, there are also some limitations that need to be considered. Metabarcoding can be highly inefficient, as non-target amplification often dominates sequencing reads when no blocking primer is applied (e.g.[39]), substantially reducing the proportion of informative reads derived from consumed prey. This limitation may be addressed by using custom blocking primers or redesigning primers to reduce non-target amplification (e.g.[40]), though both approaches require careful optimisation. Alternatively, increasing sequencing depth may help capture rare dietary items, although this approach entails higher costs[41]. Moreover, metabarcoding cannot provide information on the size of prey specimens, nor determine their life cycle stage, so it remains unclear, for example, if agamas are consuming predominantly the flying adult stages of Lepidoptera or the crawling larval stages. Poorly known endemic prey species may be missing from comparative genetic databases, meaning that potential matches with these could be overlooked, leading to an underestimation of the conservation impact of the introduced lizards. Metabarcoding results are also susceptible to amplification and taxonomic assignment biases. Employing multiple genetic markers could address some of these limitations; for instance, combining the here used trnL plant marker with an ITS2 marker would likely have enhanced taxonomic resolution and assignment success for plant taxa, albeit at additional cost. Furthermore, this study is an estimate of the diet from a single time point, which may overlook important temporal effects. For example, Rabiu[23], suggested that *A. picticauda* ate more plant material in the rainy season in its native range. Such variation could only be assessed by repeating the sampling during a different time of the year. Another potential issue with dDNA studies is the risk of "secondary predation"[42], where the DNA from prey items is detected. For instance, DNA from the thrips *Frankliniella schultzei* was detected, but these can also be consumed by beetles, and therefore, the DNA may arrive only indirectly in the pellets of the agama. Plant DNA is also widely detected in insectivorous species (e.g. Silva et al.,[43]), highlighting the need for caution in interpreting the presence of an OTU with deliberate consumption of those taxa. While this could cause some inflation in the dietary spectrum identified, the considerable number of orders and families detected demonstrates that the dietary niche of these agamas is very wide, even if they do preferentially consume ants. Finally, the potential new records of invertebrates for the island, for example, for the spider *O. putus*, still need to be confirmed – these assessments are based only on a single marker, mitochondrial DNA, and the similarity with sequences from GenBank. Errors in the published database, or evolutionary aspects such as mtDNA introgression, while unusual, mean that these results need morphological corroboration.

Conclusions

To conclude, this study shows that the dietary niche of *A. picticauda* is extremely wide, with a high number of orders and families detected. On the other hand, the diet remains remarkably conservative, with a strong preference for ants continuing in this introduced population. While the ants consumed are generally introduced, at least one endemic insect was also consumed, which raises conservation concerns that warrant further investigation. The high taxonomic levels of prey identification, often to the species level, give additional insights into the terrestrial arthropod fauna found on Réunion Island, with some potential examples of new records for the island. Plants formed a notable part of the diet, and were more frequent than previously considered. Gastrointestinal helminths were detected but could not be adequately identified due to limitations in the comparative genetic databases currently available. Still, the high diversity of genetic sequences reported in this study will provide a strong baseline for future assessments both of this introduced lizard and for other barcoding studies of the invertebrate fauna of Réunion Island.

Materials and methods

Sampling took place from June to August 2022, across 14 districts within Réunion Island (Fig. 1 and Suppl. Material Table S1). As part of an invasive species control, agamas were captured with artisanal glue traps, where the animals have been lured onto the trap with live bait (*Pycnoscelus surinamensis*). The live bait was securely contained within a transparent box, ensuring that it remained inaccessible to the lizards and was not consumed. Snout-vent length (to 1 mm) was measured with a ruler, and animals were weighed (to 0.5 g) with a Pesola scale. Since the agamas are a non-native invasive species, all captured individuals were chemically euthanised using a Lidocaine/Prilocaine cream (EMLA) applied to the mouth, and the stomach contents were immediately collected and stored in 90% ethanol and at −20 °C prior to DNA extraction. Sex was determined by examining the presence of femoral pores and hemipenial bulges and confirmed by dissection.

DNA from approximately 200 mg of each collected 66 stomach samples was extracted using an E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek, U.S.A.), following the manufacturer's instructions with a minor modification in the digestion step, using 800 µL of Gordon buffer instead of 200 µL of TL buffer to improve DNA extraction of both hard and soft tissues, following Simões et al.,[25]. Samples were vortexed and digested during 3 hours. Extracted DNA was stored at −20°C. Extraction blank samples were included to control for contaminants present in extraction kits and/or in the laboratory environment. A short fragment (~ 205 bp) of

the mitochondrial Cytochrome C Oxidase subunit I (COI) was amplified using Polymerase Chain Reaction (PCR) with the Fwh2 primers (FwhF2: 5'-GGDACWGGWTGAACWGTWTAYCCHCC-3' and FwhR2n: 5'-GTRATWGCHCCDGCTARWACWGG-3') from Vamos et al.,[44], which are among the most effective primers for terrestrial arthropods[45], and have worked well in other insectivorous lizards (e.g.[25]). To assess plant consumption, the g (5'- GGGCAATCCTGAGCCAA-3') and h (5'- CCATTGAGTCTCTGCACCTATC-3') primers[46] were used targeting the short P6-loop of chloroplast trnL (UAA) intron (up to 143 bp). The primers were modified to include Illumina adaptors and a 0–5 bp addition of N bases between the adaptor and the primer to increase sequencing diversity and quality. The different primer variations were then combined before PCR reactions, resulting in mixed forward and reverse primer single solutions.

The PCR reactions for both plant and animal amplifications consisted of 5 µL of QIAGEN Multiplex PCR Master Mix (Qiagen, Crawley, UK), 0.3 µL combination of six Forward primers, 0.3 µL combination of Reverse Primers, 2.4 µL of ultra-pure water, and 2 µL of DNA extract. Cycling conditions used consisted of an initial denaturation at 95 °C for 15 min, followed by 45 cycles of 95 °C for 30 s, annealing at 52 °C for 45 s, extension at 72 °C for 20 s, and a final extension at 60 °C for 5 min. Negative controls were included to detect possible contamination.

Initial PCR clean-up to remove unused primers and primer dimers was performed employing the Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA), with a proportion of 0.85 µL of magnetic beads to 1 µL of PCR product. To attach unique barcodes and Illumina sequencing indexes to each sample, a second PCR was performed using a distinct combination of index sequences per sample. This PCR was performed using 2.8 µL of ultra-pure water, 7µL of 2x Kapa HiFi, 1.4 µL of each index, and 2.8 µL of cleaned DNA. Indexing the PCR required an initial denaturation of 95 °C for 3 min, followed by 10 cycles of 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension of 72 °C for 5 min. PCR products were examined in a 2% agarose gel, knowing that amplicons should be ~ 100 bp longer than in the previous PCR. A second PCR clean-up was then performed, under the same conditions as before, except that the beads' ratio was 0.8.

After the second PCR clean-up, all indexed PCRs were quantified using an Epoch™ Microplate Spectrophotometer (BioTek Instruments, Inc.; Winooski, VT, USA), followed by normalisation to obtain the same concentration in all the samples, after which the samples were pooled. This pool was quality tested in a TapeStation 4200 High Sensitivity D1000 Assay (Agilent, Santa Clara, CA), and a cleaning was performed using a 0.78 ratio of beads. A second quality control test in the TapeStation confirmed the success of the cleaning. The final pool was sent to GENEWIZ Next Generation Sequencing laboratory with a final concentration of 27nM to be sequenced in an Illumina MiSeq sequencer with 2 × 250 bp paired-end (PE) configuration, and PhiX (≤ 30%) was used to increase sequencing diversity.

Data analysis

Bioinformatic processing of sequencing reads was done separately for each molecular marker. The software PEAR[47] was used to merge the forward and reverse reads into single sequences, discarding single and unassembled reads, as well as cases with less than 26 bp of overlap. This overlap threshold was selected to accommodate the relatively short amplicon lengths used in this study, especially for trnL. It also maximises read retention while ensuring reliable merging. PEAR is designed to handle variable and short overlap lengths and does not require long fixed overlaps for accurate merging[47]. Primers were removed, using the command *ngsfilter* from OBITools[48], reads were counted to check for successful rates of the previous step with the *grep* command, and dereplicated into unique sequences using the *obiuniq* command. Sequence cleaning was performed to remove sequences with less than 10 reads and chimeras, using the *obiclean* command, and those with a length between 202 and 208 bp (COI) and 30 to 120 bp (trnL) were kept and clustered at a 99% similarity threshold to form Operational Taxonomic Units (OTUs) using VSearch[49]. OTUs that were represented by a read count < 1% of the total number of reads obtained for each sample were removed, as were all reads identified in the extraction and PCR controls of the corresponding sample batch.

The obtained OTU table and sequences were further cleaned to remove redundant and unreliable OTUs using the R package LULU[50], and the taxonomic assignment of OTUs was performed using BOLDigger[51], followed by manual inspection and curation. We used the BLAST function from NCBI implemented in Geneious Prime 2021.1.1[52] when species-level assignments were not possible with BOLDigger. If similarity was > 95% assignment was made to the species level, or genus level in cases where more than one species from the same genus had a > 95% match. Assignment was made to the family level if between 90 and 95%, and to the class level if below 90% similarity. Prey OTUs that were represented by a read count < 1% of the total number of diet reads were removed. Additionally, all reads identified in the extraction and PCR controls were subtracted from the corresponding sample batch.

For the statistical analysis, R 4.1.2 (R Core Team 2022) was used to assess differences in dietary descriptors (i.e., diet richness and composition) between localities and sexes. The log-transformed body size (SVL) and mass (weight) variation between sexes across localities was summarised using boxplots. Body condition was assessed by investigating the relationship between body mass and body length in both males and females through a linear regression.

Dietary analysis was based on two different taxonomic levels: OTU (all taxonomic units identified to the highest resolved taxonomic level) and family. For the OTUs not identified to the species level, we built a neighbour-joining tree in Geneious Prime v. 2024.0.2 (Biomatters), visually inspected the corresponding alignment, and checked for patterns of genetic similarity (~ 98%) in order to cluster them into distinct taxa (e.g., Coleoptera 1, Coleoptera 2, and so on). The frequency of occurrence (FO) of each food item was calculated as the number of occurrences of the diet item (maximum 1 occurrence per sample) divided by the total number of sequenced reptile stomach samples.

To assess the effects of locality, sex and size on the average number of prey taxa detected per sample (i.e., richness), a General Linear Model (GLM) was implemented. For this, the function *glm* was used and its significance tested with the *anova* function, both from the car package[53], fitting a Poisson error distribution. Graphical representation of the relationships depicted from the GLM results was performed using the *effect* function from the effects R package[54], where richness means and standard errors were derived from the model's parameter estimates. Pairwise comparisons were performed with the function emmeans (with a Tukey adjustment) from the package emmeans[55].

To calculate the dietary niche-width between the sexes and among the different populations, prey rarefaction and extrapolation curves were built using the R package iNEXT v. 2.0.20[56]. Analyses were conducted with incidence frequencies for prey taxa. We compared the estimated richness considering completeness (i.e., sample coverage) instead of sample size (i.e., number of samples), to avoid biases of communities with different levels of richness requiring different sampling efforts in order to be sufficiently characterised[57]. Considering that the 95% confidence interval is a very conservative approach, we considered that differences were significant if the 84% confidence interval (a proxy for $\alpha = 0.05$) of both estimates did not overlap[58].

Permutational multivariate analysis of variance (PerMANOVA) was used to compare the OTU and family diet composition between the sexes and the different localities with the vegan R package (function *adonis2*[59]. Presence or absence of each prey item in each sample was used to build a Jaccard dissimilarity matrix using the *vegdist* function from the R package vegan. A homogeneity of dispersion test (function *betadisper*) was also carried out to assess if the observed differences in PerMANOVA could be due to unequally dispersed values across the different groups[60].

Data availability

The datasets generated and/or analysed during the current study are available in the European Nucleotide Archive (ENA) repository, under project PRJEB114936.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval
The invasive species control programme follows order no. 2023-605 SG/SCOPP/BCPE in accordance with Article L411.8 of the Environment Code on the territory of Réunion Island. Access and benefit-sharing was approved in accordance with Article17, paragraph 2, of the Nagoya Protocol and is accessible under the reference number TREL2206915S/624.

Additional information
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