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Rediscovering the lost: eDNA detection of freshwater decapods on Réunion Island

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Abstract – Freshwater ecosystems on tropical oceanic islands host unique biodiversity. On Réunion Island, freshwater decapod crustaceans are key indicators of ecosystem health, yet their distribution and conservation status remain poorly documented. This study aimed to investigate the presence of the endemic prawn *Macrobrachium hirtimanus*, historically reported but unconfirmed for decades, while producing the first island-wide molecular inventory of freshwater decapods using environmental DNA (eDNA) metabarcoding. Water samples (4 L) were collected from 19 riverine sites, and eDNA was extracted and amplified using mitochondrial 16S rRNA primers validated for crustacean detection, followed by high-throughput sequencing. Bioinformatics analyses yielded 250,545 reads and 198 operational taxonomic units (OTUs), representing diverse amphidromous freshwater, coastal marine, and terrestrial decapods. Species richness was largely uniform across sites, though Rivière des Roches, Sainte-Marie, and Saint-Denis hosted higher diversity. Beta diversity showed strong spatial structuring among watersheds, driven primarily by species turnover. Widespread taxa included *Atyoida serrata*, *Caridina typus*, and *Macrobrachium australe*, while *C. henriettae* and *Halocaridinides* sp. were more localized. *M. hirtimanus* was not detected, consistent with its current absence, though continued targeted surveys are required before any formal reassessment. Overall, this study demonstrates the utility of eDNA metabarcoding for monitoring freshwater biodiversity and informing conservation planning on tropical islands.

Keywords: Decapod / environmental DNA / freshwater / tropical island / amphidromous

1 Introduction

Freshwater ecosystems on tropical islands harbor a unique and often fragile biodiversity, yet they remain understudied compared to terrestrial and marine environments (Falkland, 2002). In the southwest Indian Ocean, Réunion Island, a young volcanic territory (c. -2 Ma, Chevallier and Vatin-Perignon, 1982) with steep topography and a limited number of torrential rivers, hosts a range of crustacean freshwater species (Keith, 2002). Due to the island's geographic isolation, located approximately 180 km east of Mauritius and 700 km from Madagascar, Réunion's freshwater fauna has evolved significant genetic distinctiveness, with high levels of endemism and local adaptation (Myers *et al.*, 2000; Thébaud *et al.*, 2009).

Among the most ecologically significant members of these communities are freshwater decapod crustaceans, including shrimps and crabs. Many are amphidromous and depend on the unimpeded connectivity between rivers and the sea to complete their life cycles (Keith, 2002; McDowall, 2010). These organisms play an essential role in tropical aquatic ecosystems as detritivores, algal grazers, sediment bioturbators, and prey for fishes and birds (Bauer, 2013; Reynolds *et al.*, 2013; Szaniawska, 2018). They contribute to nutrient cycling, primary productivity, and the structural integrity of streambed habitats. In particular, amphidromous decapods are valuable indicators of watershed health and ecological connectivity due to their reliance on both freshwater and marine environments (Teichert *et al.*, 2025).

In Réunion Island, native freshwater decapods include several species from the families Atyidae, such as *Atyoida serrata* (Spence Bate, 1888) and *Caridina typus* (H. Milne-Edwards, 1837); Palaemonidae including *Macrobrachium australe* (Guérin-Méneveille, 1838) and *M. hirtimanus* (Olivier, 1811); and Varunidae represented by *Varuna litterata*

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(Fabricius, 1798), among others (Keith, 2002; UICN France, 2016). While some species show ecological plasticity and broad distributions, others are narrowly endemic and particularly vulnerable to environmental disturbances. Notably, the "betangue prawn" *Macrobrachium hirtimanus*, endemic to the Mascarene Islands, and once common in several rivers on the island, has not been observed since the 1980s and is now considered possibly extinct (De Grave, 2013). This species was historically recorded in the Rivière des Marsouins, Rivière Langevin, and Rivière du Mât (Kiener, 1981; Keith and Vigneux, 2000). Its decline coincided with the arrival of the relatively morphologically similar *Macrobrachium lepidactylus* (Hilgendorf, 1879), a species also indigenous to the southwest Indian Ocean, that may have displaced it through ecological competition or niche overlap (Keith and Vigneux, 2000). The absence of recent records of *M. hirtimanus* has raised concerns among scientists and managers alike. In 2023, the Réunion Basin Committee via the Aquatic Environments Commission emphasized the urgent need to clarify the species' status, either to identify remnant populations or to confirm its local extinction.

This urgency extends beyond a single species. Anthropogenic pressures, such as pollution, sedimentation, urban runoff, and the construction of dams or weirs, have disrupted natural flow regimes and migration corridors essential to amphidromous species (Jarvis and Closs, 2019; Williams-Subiza and Epele, 2021). Additionally, the introduction of non-native species poses growing threats. For example, the recent invasion of *Neocaridina davidi* (Bouvier, 1904), a freshwater shrimp native to East Asia, has been documented in Réunion's aquatic systems, raising concerns over potential competition, habitat alteration, and disease transmission (Prati *et al.*, 2024).

In this context, a comprehensive reassessment of the island's freshwater decapod fauna is both timely and essential. Recent molecular studies in the Indo-Pacific have revealed high levels of cryptic diversity among decapod crustaceans, highlighting the limitations of morphology-based taxonomy alone (de Mazancourt *et al.*, 2021). Improved detection tools are now available, with environmental DNA (eDNA) metabarcoding offering a powerful and non-invasive method to identify aquatic species from trace genetic material left in water, including faeces, mucus, gametes, or decomposing tissue (Veilleux *et al.*, 2021).

This method has already proven effective for aquatic biodiversity surveys in Réunion (Jannel *et al.*, 2024) as we possess a reference library of DNA sequences from accurately identified specimens. Here, we applied eDNA metabarcoding targeting the mitochondrial 16S rRNA gene, a marker commonly used for decapod identification due to its high interspecific resolution and relative sequence conservation (Meyer, 1994; Calo-Mata *et al.*, 2009; Barua *et al.*, 2021). Our primary goal was to assess whether *M. hirtimanus* could still be detected in Réunion's freshwater ecosystems using molecular tools. In parallel, this survey provided an updated and comprehensive overview of the island's freshwater decapod diversity, offering new insights into both native and non-native species distributions.

2 Methods

2.1 Study sites

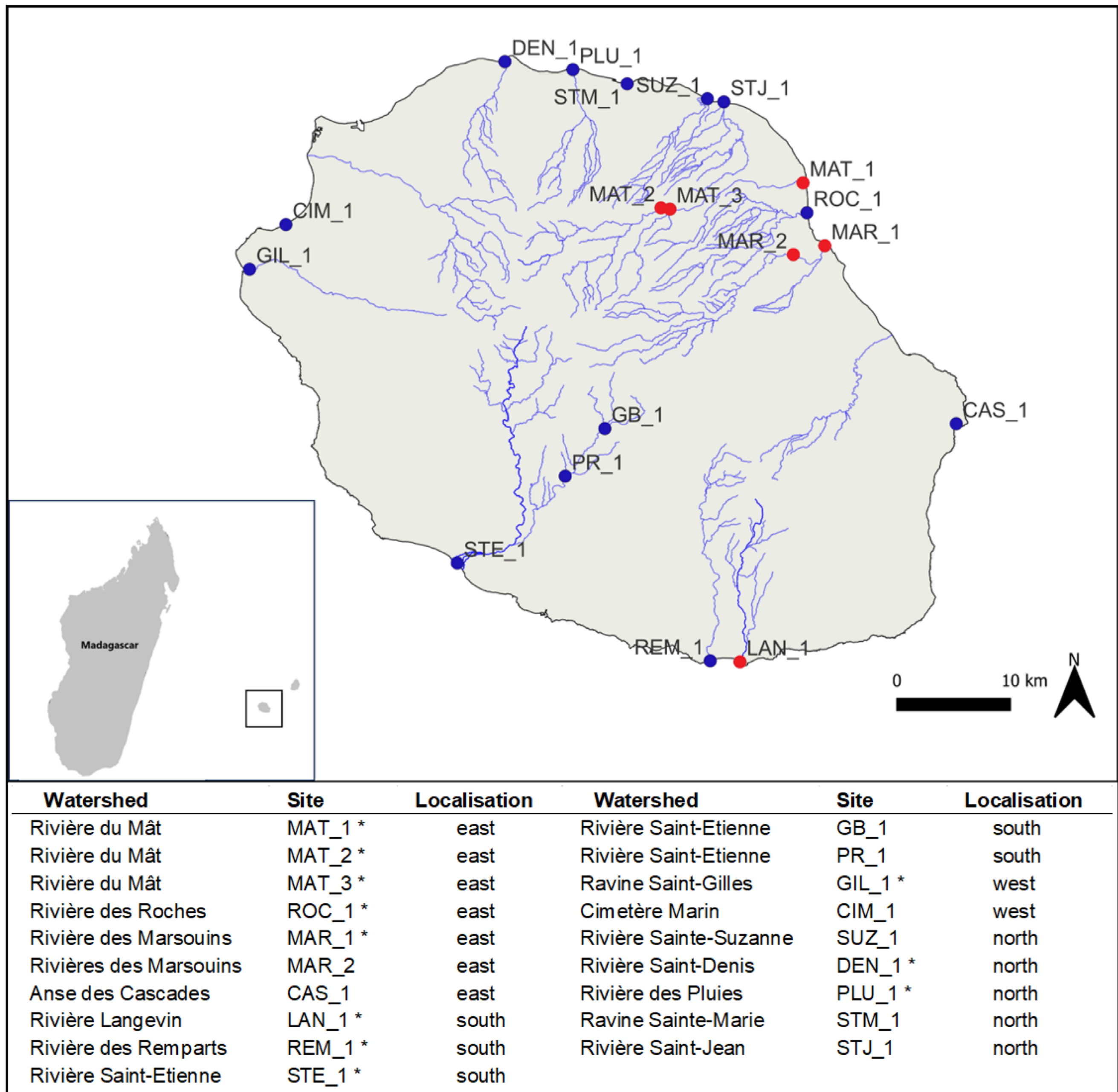
A total of 19 sampling stations were selected across Réunion Island to include both historical locations of *Macrobrachium hirtimanus*, notably in the Rivières du Mât, des Marsouins, and Langevin, and additional sites with similar hydrological characteristics to maximize the probability of detecting *M. hirtimanus*, while also capturing representative freshwater decapod assemblages for broader ecological assessment (Fig. 1). These included comparable flow regimes, habitat types, and community structures likely to support amphidromous crustaceans and opportunistic inventories in parts of apical and preserved watersheds.

To strengthen detection reliability and account for potential temporal variation in eDNA signals, such as those caused by hydrological conditions or life cycle dynamics, 11 of the 19 stations were sampled twice (Fig. 1). Two sampling campaigns were conducted: the first in July 2022 and the second in December 2024. The July campaign corresponded to the austral dry season, when water levels are stable and DNA degradation rates are reduced, minimizing signal dilution. The December campaign, scheduled during the wetter season, was intended to represent contrasting environmental conditions; however, exceptionally low rainfall during that period resulted in hydrological conditions like those of the dry season. The two-year interval was therefore not designed to assess interannual or seasonal ecological variation, but rather to evaluate the reproducibility of detections and the robustness of community patterns across distinct sampling conditions.

2.2 Sampling methodology

A total of 30 environmental DNA samples were analyzed, corresponding to 30 unique sampling stations (19 sampled in 2022 and 11 resampled in 2024). At each site, three field replicates were collected and processed independently through DNA extraction, amplification, and bioinformatic assignment to ensure reproducibility. For ecological and diversity analyses, replicate data were pooled at the station level, resulting in one representative eDNA sample per site (Fig. S1).

The water sampling protocol was modified from Majaneva *et al.* (2018), and according to the recommendations of Pawlowski *et al.* (2020). Briefly, the steps are as follows: at each sampling site, three replicates of water samples (1 site =3 replicates) were collected from the same location using 5-litre cans previously decontaminated with a 50% bleach solution and single-use gloves. The samples were placed in coolers with ice during transport from the study site to the laboratory. Filtration was carried out within 6 h of collection. All procedures were carried out on a bench cleaned with a 50% bleach solution, and the equipment was decontaminated between each filtration. Using a vacuum pump, the water was filtered through magnetic funnels onto filters made from a mixture of cellulose esters (MCE: nitrate and acetate; Merck Millipore; 47 mm diameter; 0.45 μm pores). Following Peixoto *et al.* (2021), the water was filtered until the filter membrane clogged. An average of 3310 ± 1072 ml was filtered per replicate (minimum 1250 ml and maximum 4000 ml). A filtration control was performed (*i.e.*, two liters of cleaning laboratory water) and was used to eliminate any possible contamination from the filtration technique. Following



Watershed	Site	Localisation	Watershed	Site	Localisation
Rivière du Mât	MAT_1 *	east	Rivière Saint-Etienne	GB_1	south
Rivière du Mât	MAT_2 *	east	Rivière Saint-Etienne	PR_1	south
Rivière du Mât	MAT_3 *	east	Ravine Saint-Gilles	GIL_1 *	west
Rivière des Roches	ROC_1 *	east	Cimetère Marin	CIM_1	west
Rivière des Marsouins	MAR_1 *	east	Rivière Sainte-Suzanne	SUZ_1	north
Rivières des Marsouins	MAR_2	east	Rivière Saint-Denis	DEN_1 *	north
Anse des Cascades	CAS_1	east	Rivière des Pluies	PLU_1 *	north
Rivière Langevin	LAN_1 *	south	Ravine Sainte-Marie	STM_1	north
Rivière des Remparts	REM_1 *	south	Rivière Saint-Jean	STJ_1	north
Rivière Saint-Etienne	STE_1 *	south			

Fig. 1. Sampling site locations in Réunion Island (southwestern Indian Ocean). The stations marked in red correspond to rivers historically colonized by *Macrobrachium hirtimanus*. Stations marked with an asterisk correspond to double sampling (C1: July 2022 and C2: December 2024).

Allison *et al.* (2020), the filters were stored in 2-ml tubes containing silica beads, which dry out the filters and prevent DNA degradation. The sample filters were then stored at −20°C until extraction. The DNA was extracted from the filters using a protocol modified from the DNeasy Blood and Tissue DNA extraction kit (Qiagen, Hilden, Germany) to include a bead-beating pre-treatment step (Closek *et al.*, 2019), with an elution volume of 100 µl. Two laboratory controls were performed at the same time as the sample extraction (*i.e.*, one laboratory control per campaign), performing an extraction without a filter. The laboratory controls were analyzed separately. The samples were stored at −20°C until sequencing.

2.3 16S rRNA Illumina sequencing and bioinformatic analyses

Amplification and high-throughput sequencing were conducted according to the protocols of the ADNid-Qualtech laboratory (ADNid, Monteferrier-sur-Lez, France). A fragment of the 16 S rRNA gene (~ 520 bp) was amplified by PCR using primers 16Sa-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sb-H2 (5'-CTCCGGTTTGAACTCAGATCA-3') (Palumbi, 1996). DNA amplification was performed in 25 µl PCR reactions, containing approximately 3 ng of template DNA, 2.5 mM MgCl 2,0.26 mM of each nucleotide, 0.3 µM of each

primer, 5% DMSO, 1 ng of BSA and 1.5 units of QBIOTAQ polymerase (MPBiomedicals). Amplification products were generated by an initial denaturation step of 4 min at 94°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s, extension at 72°C for 60 s and a final extension step at 72°C for 7 min. Each eDNA extract underwent a single target amplification (PCR1), with quality control performed on an 2% agarose gel. A second PCR (PCR2) was then carried out for dual indexing (UDI) and library preparation, followed by pooling of libraries in equimolar quantities. Qualitative and quantitative controls were performed using a Fragment Analyzer ™ (Agilent Technologies Inc., Santa Clara, USA). Libraries were deposited on MiSeq v3 flow cells and sequenced on the Illumina MiSeq platform (2 × 300 bp format), providing a sequencing depth of at least 100,000 reads per sample. All sequencing runs included negative controls (field blanks, extraction blanks, and PCR blanks).

Bioinformatics analyses were performed using the FROGS pipeline according to Escudié *et al.* (2018) using default parameters. Each replicate was treated individually. A first quality/filtration step was performed, to remove primers, short sequences (<100 bp), ambiguous bases. Sequences were clustered using the SWARM method with a maximum aggregation distance of one difference (d=1) (Mahé *et al.*, 2014). These sequence groups are referred to as OTUs (Operational Taxonomic Units) throughout the manuscript for consistency, although they are generated using a clustering approach that considers one difference per aggregation step. Chimera detection and elimination were based on VSEARCH with the *de novo* UCHIME method (Edgar *et al.*, 2011; Rognes *et al.*, 2016). FROGS guidelines recommend applying an abundance filter prior to the taxonomic affiliation process (Escudié *et al.*, 2018). Specifically, OTUs with a relative read abundance (RRA) below 0.02% within a sample were excluded. This conservative cutoff reduces noise from sequencing errors or tag-jumping while preserving true low-frequency taxa (Escudié *et al.*, 2018). To control for potential contamination (false positives or sequencing errors), negative controls (field blanks, extraction blanks, and PCR negatives) were included at every step. For each OTU, the total number of reads detected in any control was subtracted from the corresponding environmental counts before downstream analyses. Representative OTU sequences were initially compared against the GenBank nucleotide database at the National Centre for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov) using BLAST. This preliminary step provided a broad overview of all amplified taxa. Following this initial assignment, only OTUs affiliated with the order Decapoda were retained for further analysis. All non-decapod OTUs (*e.g.*, insects, amphibians, bacteria) were excluded to focus specifically on the target taxonomic group. These retained Decapoda OTUs were then re-examined using a curated local reference database built from validated sequences of Mascarene Archipelago, primarily from Réunion and Mauritius, crustaceans (Natural History Museum of Paris). Taxonomic assignments were refined using both percentage identity and query coverage criteria: OTUs with ≥97% identity and ≥97% query coverage were assigned to the species level. OTUs with ≥95% but <97% identity (and ≥95% query coverage) were conservatively assigned to the genus level. OTUs with <95% identity were not taxonomically assigned beyond higher ranks (family or order). This hierarchical assignment scheme ensures that only robust matches are interpreted at the species level, reducing false positives and overclassification (Gibson *et al.*, 2015; Elbrecht *et al.*, 2017). The OTU sequences were also compared with the *M. hirtimanus* reference sequence obtained by extracting DNA from historical collection specimens (de Mazancourt *et al.*, *In press*).

2.4 Statistical analyses

Statistical analyses were conducted using R 4.3.1 (R Core Team, 2024) with data processing performed using the Phyloseq package (McMurdie and Holmes, 2012, 2013). The Phyloseq objects were utilized for subsequent analyses. Diversity analyses were carried out using the Vegan package (Oksanen *et al.*, 2022). For subsequent ecological analyses, replicate data were pooled at the station level (three samples per site) to provide an integrated estimate of local species presence. A taxon was considered present at a given station if detected in at least one of the three field replicates. This criterion was adopted to maximize detection probability for rare or low-abundance taxa, for which eDNA signals can be spatially heterogeneous and inconsistently detected among replicates.

To evaluate whether sequencing depth was sufficient to capture most of the crustacean diversity, coverage-based rarefaction and extrapolation analyses were performed using the iNEXT package (Hsieh *et al.*, 2016). These analyses estimated sample completeness (sample coverage) and asymptotic species richness (Chao *et al.*, 2014) based on Hill numbers (q=0).

First, we studied crustacean communities' diversity (alpha diversity) using the specific richness and Shannon index. Alpha diversity indices were calculated using incidence (presence/absence) data rather than read counts, to limit potential biases related to amplification efficiency or sequencing depth across samples. Shannon index was calculated for completeness, but with incidence data, it primarily reflects richness, and evenness cannot be independently assessed. An assessment of ecological diversity between the different watershed and sampling campaigns was conducted using a Kruskal-Wallis nonparametric test and Wilcoxon test.

Secondly, we studied community structuring with beta diversity. Beta diversity analyses were conducted using the Jaccard dissimilarity index based on presence/absence data (binary form). Differences in community composition were tested using permutational multivariate analysis of variance (PERMANOVA, adonis2 function), with 999 permutations constrained within sampling sites to account for repeated sampling between campaigns. Homogeneity of multivariate dispersion among groups was assessed using the betadisper function. Community structure was represented using Principal Coordinates Analysis (PCoA) based on Jaccard dissimilarity distances. The species beta diversity partitioning proposed by Baselga, (2010) was used. This approach was based on three multi-site different dissimilarity coefficients: (1) Jaccard coefficient, a measure of overall beta diversity, (2) turnover coefficient, a measure of turnover without influence of richness differences, and (3) nestedness coefficient,

measuring nestedness resulting from richness differences (Baselga, 2010; Legendre, 2014).

Finally, we studied composition of the crustacean communities. These analyses were conducted at the species level.

3 Results

A total of 1,707,984 reads were obtained from the 30 eDNA samples collected (after pooling replicates), corresponding to 1,185 OTUs (Tab. S1). Of these, 214 were affiliated with bacteria, viruses, or sequences of uncertain/mixed taxonomy and were excluded. The remaining 971 OTUs corresponded to eukaryotic sequences, primarily Mollusca, Annelida, or Arthropoda. After quality filtering, chimera removal, and retention of OTUs associated with the Decapoda order, 198 OTUs were retained corresponding to 250,545 reads, with an average of 8,331 ± 8862 reads per sample (minimum 10 reads and maximum 28,816 reads). Rarefaction curve showed an early stationary phase indicating sufficient sequencing depth to capture most of the crustacean diversity (Fig. S2). Taxonomic assignment of these OTUs identified representatives from 6 decapod families, 10 genera, and 11 species (Tab. 1). Three taxa could not be assigned to species level. These corresponded to *Halocaridinides sp.*, *Macrobrachium sp*., and *Metapenaeus sp*. This was either due to the absence of corresponding reference sequences in public or local databases, or because the sequences were too degraded to allow for confident identification.

Coverage-based rarefaction and extrapolation (iNEXT; q=0) showed that sampling completeness was high at the island scale, with sample coverage reaching 0.98 at the observed sequencing depth (Fig. S3). The asymptotic richness (S.chao1=198 OTUs) was identical to the observed value (S.obs =198 OTUs), indicating that sequencing depth was sufficient to capture most detectable crustacean diversity.

3.1 Diversity and disappearance: crustacean species composition and the case of *Macrobrachium hirtimanus*

Crustacean OTUs recovered from eDNA samples were compared to a reference sequence of *Macrobrachium hirtimanus*. Despite targeting historical sites (Rivière du Mât, Rivière des Marsouins et Rivière Langevin, Fig. 1), none of the OTUs could be confidently assigned to *M. hirtimanus*. Similarly, *Macrobrachium lepidactylus*, a morphologically close species known from traditional surveys, was not detected.

A total of 14 taxa were identified across the 30 environmental inventories. Among these, four were non-freshwater species. The marine-associated taxa *Metapenaeus sp.* and *Penaeus indicus* (H. Milne-Edwards, 1837) were only detected at the Rivière des Remparts station during the first sampling campaign (C1_REM_1). The terrestrial crab *Geograpsus grayi* (H. Milne-Edwards, 1853) was found at the same station during the second campaign (C2_REM_1). The species *Cardisoma carnifex* (Herbst, 1796) was uniquely detected at Rivière Sainte-Marie (C1_STM_1).

The remaining 10 taxa were amphidromous freshwater species, affiliated with three main crustacean families: Atyidae, Palaemonidae, and Varunidae. Within the Atyidae, five species were identified (Tab. 1). *Atyoida serrata* and *Caridina typus* were widely distributed across multiple rivers on the island. *Atyoida* cf. *serrata* was present at four distant sites: SaintGilles (GIL_1), Bras de Caverne in Rivière du Mât (MAT_3), Anse des Cascades (CAS_1), and Langevin (LAN_1). *Caridina henriettae* (de Mazancourt, Mennesson, Marquet and Keith, 2025) was restricted to north-eastern stations including MAT_2, ROC_1, DEN_1, STM_1, PLU_1, SUZ_1, and STJ_1, as well as the western station at Saint-Gilles (GIL_1). *Halocaridinides sp.* was observed in the rivers of Saint-Denis (DEN_1) and Roches (ROC_1).

In the Palaemonidae family, three species and several unassigned *Macrobrachium* OTUs were detected (Tab. 1). *Macrobrachium australe* occurred widely across the island. *Macrobrachium lar* (Fabricius, 1798) was found only at the mouth of Rivière du Mât (MAT_1) and Anse des Cascades (CAS_1). *Palaemon concinnus* (Dana, 1852) was detected solely at the Saint-Étienne estuary (STE_1). Unassigned *Macrobrachium* were present at Saint-Denis (DEN_1), Les Roches (ROC_1), and Saint-Étienne (STE_1).

Only one species from the Varunidae family was recorded (Tab. 1). *Varuna litterata* was detected exclusively at the station located in the marine cemetery of Saint-Paul (CIM_1).

3.2 Limited variation in local diversity: alpha diversity patterns across river sites

Kruskal-Wallis tests revealed no significant differences in alpha diversity among river sites when assessed at the OTU level, using either Shannon diversity (χ^2=23.75, df=18, p=0.163) or Observed richness (χ^2=19.20, df=18, p=0.378). Although not statistically significant, the difference in test statistics suggests that OTU-level data may capture finer-scale variation in community structure, potentially reflecting intra-species diversity or cryptic lineages detectable through eDNA. When diversity was analysed at the species level using presence/absence data, both Shannon and Observed indices produced identical results (χ^2=21.24, df=18, p=0.268), reflecting that Shannon, in this context, behaves largely as a richness-like metric and does not independently capture evenness. For example, sites such as Rivière des Roches (ROC_1), Sainte-Marie (STM_1), and Saint-Denis (DEN_1) showed higher species richness (averaging 5.5 species), while sites like the upper Marsouins River (MAR_2) and Petite Ravine (PR_1) displayed minimal richness, with only a single species detected on average (Fig. 2). In addition, comparisons between the two sampling campaigns (2022 and 2024) revealed no significant effect of sampling period on alpha diversity, whether assessed by Shannon index or observed richness (Wilcoxon test, W=98.5, p=0.808). This indicates that overall local diversity patterns were temporally consistent across campaigns.

3.3 Distinct regional assemblages: spatial structuring of crustacean communities

Beta diversity analyses (PERMANOVA, 999 permutations constrained within sites) revealed a significant effect of watershed on community composition (R^2_watershed =0.61, F =1.46, p=0.05), indicating strong spatial structuring of crustacean assemblages across rivers. In contrast, the sampling

Table 1. Summary of crustacean families and species detected by environmental DNA (eDNA) metabarcoding across sampling stations on Réunion Island. Crosses indicate detections. “C1” and “C2” refer to the two sampling campaigns (C1: July 2022 and C2: December 2024). Taxa are grouped into three ecological categories: amphidromous, marine, and terrestrial.

		Amphidromous										Marine			Terrestrial	
		Atyidae					Palaemonidae				Varunidae	Penaeidae		Gecarcinidae	Grapsidae	
Watershed	Site	*Atyoida serrata*	*Atyoida cf. serrata*	*Caridina henriettae*	*Caridina typus*	*Halocaridinides sp.*	*Macrobrachium australe*	*Macrobrachium lar*	*Macrobrachium sp.*	*Palaemon concinnus*	*Varuna litterata*	*Metapenaeus sp.*	*Penaeus indicus*	*Cardisoma carnifex*	*Geograpsus grayi*	*Species Richness*
Rivière du Mât	C1_MAT_1	X														1
	C1_MAT_2	X														1
	C1_MAT_3	X														1
	C2_MAT_1						X	X								2
	C2_MAT_2			X												1
	C2_MAT_3	X	X													2
Rivière des Marsouins	C1_MAR_1	X			X		X									3
	C1_MAR_2	X														1
	C2 MAR_1	X		X			x									3
SaintEtienne	C1_STE_1						X									1
	C2_STE_1	X			X		X		X	x						5
	C1_GB_1	X														1
	C1_PR_1	X														1
Ravine Saint-Gilles	C1_GIL_1	X	X	X	X		X									5
	C2_GIL_1	X			X		X									3
Rivière des Roches	C1_ROC_1	X		X	X		X									4
	C2_ROC_1	X		X	X	X	X		X							6
Rivière Saint-Denis	C1_DEN_1	X		X	X	X	X									5
	C2 DEN_1			X		X	X									3
SainteMarie	C1 STM 1			X	X		X							X		4
Langevin	C1_LAN_1	X	X		X		X									4
	C2 LAN 1	X	X				X									3
Remparts	C1_REM_1	X										X	X			3
	C2_REM_1	X													X	2
Rivière des pluies	C1_PLU_1	X		X	X		X									4
	C2_PLU_1	X					X									2
SainteSuzanne	C1_SUZ_1			X	X		X									3
Saint-Jean	C1 STJ 1			X			X									2
Anse des Cascades	C1_CAS_1	X	X		X			X								4
Saint-Paul	C1_CIM_1				X		X				X					3

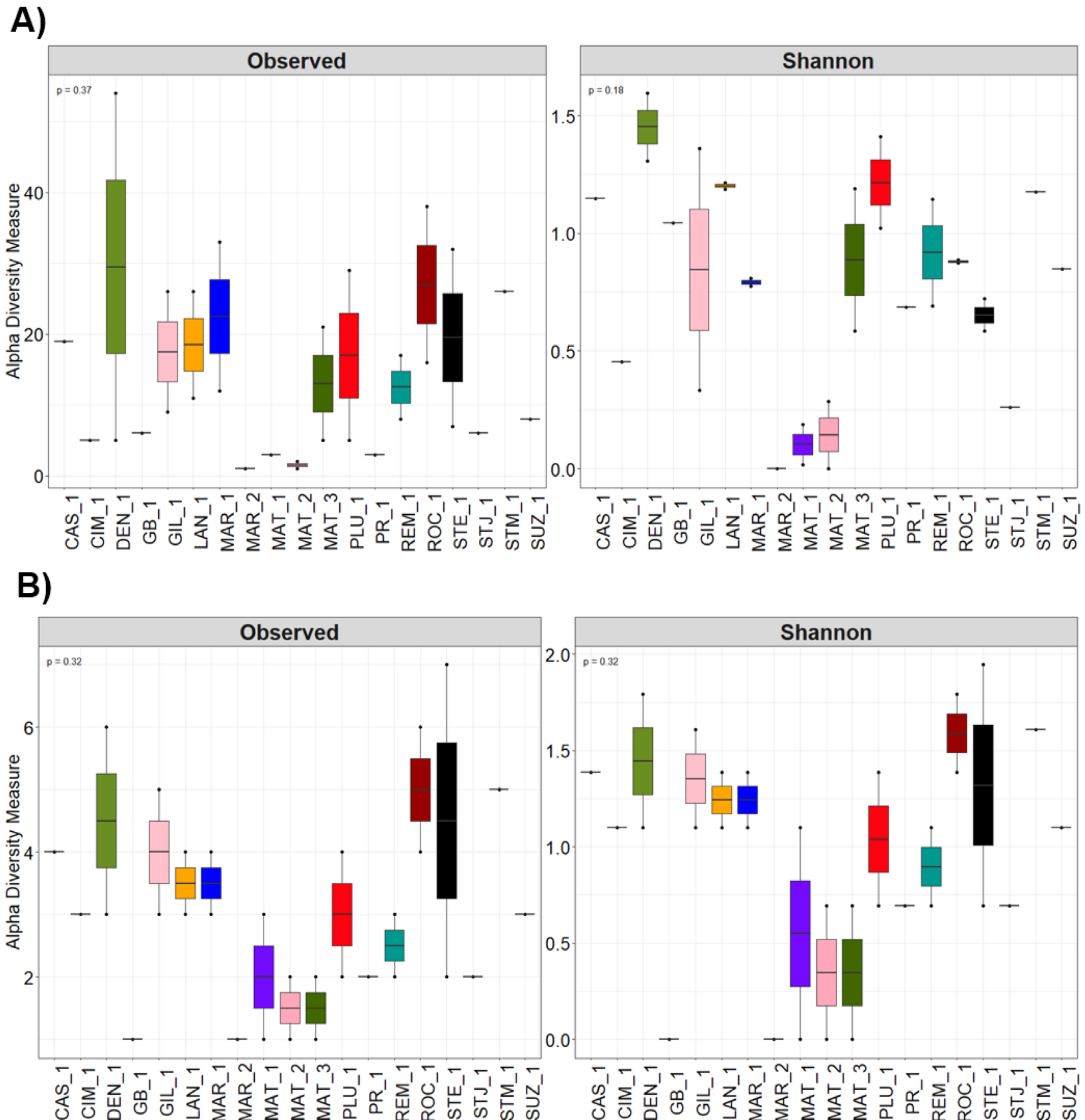


Fig. 2. Alpha diversity across sampling stations, expressed as Observed Richness and Shannon Diversity Index at (A) the Operational Taxonomic Unit (OTU) level and (B) the species level.

campaign factor showed no significant effect on assemblage composition (R^2 campaign =0.02, F=0.69, p=0.612), confirming the temporal consistency of community patterns between the 2022 and 2024 surveys. Permutations were constrained within sites (999 permutations) to respect repeated measures. Homogeneity of multivariate dispersion among groups was verified (betadisper test: F=1.46, p=0.543), indicating that observed compositional differences were not driven by unequal dispersion. Ordination via Principal Coordinates Analysis (PCoA, Fig. 3) further illustrated this pattern, with sampling sites clustering according to their geographical location around the island. Notably, communities in northern rivers differed significantly from those in the south (R^2 north-south= 0.27, F=4.73, p=0.01) and east (R^2 north-east =0.18, F=3.87, p=0.008). This pattern was confirmed by beta diversity partitioning based on the Jaccard index, which indicated a high overall dissimilarity among sites (beta.JAC =0.942). Most of this beta diversity was attributed to species turnover (beta.JTU =0.787), rather than nestedness (beta.JNE =0.155).

4 Discussion

This study provides the first comprehensive application of eDNA metabarcoding to assess the diversity and distribution of freshwater decapod crustaceans on Réunion Island. The results confirm the method's potential to detect a broad

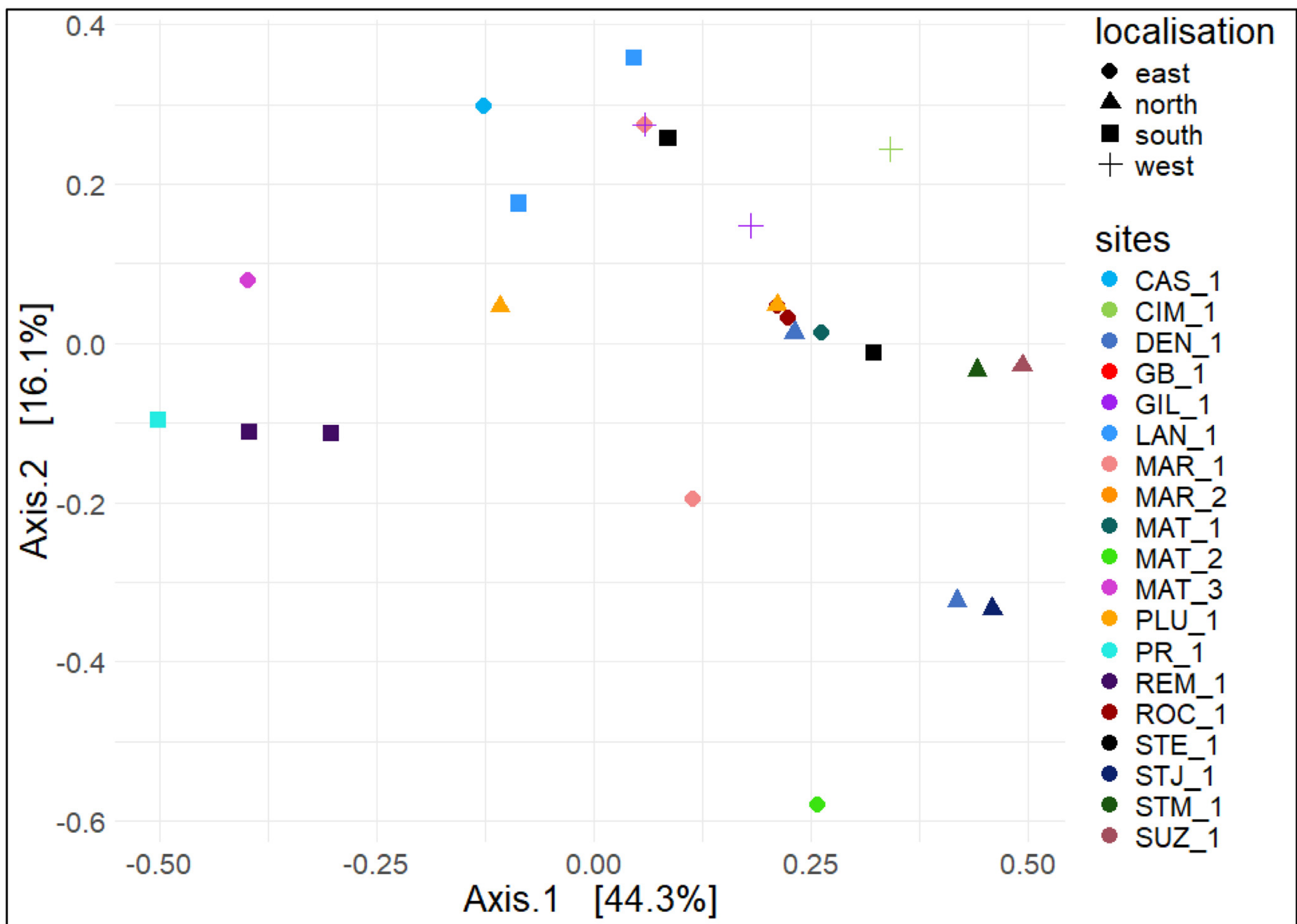


Fig. 3. Beta diversity of crustacean communities across sites, visualized using Principal Coordinates Analysis (PCoA) based on Jaccard dissimilarity indices, illustrating spatial variation around Réunion Island.

range of taxa, including expected native species (10 taxa, including *Atyoida* cf. *serrata* or *Macrobrachium australe*), new regional records (2 taxa, *i.e.*, *Atyoida* cf. *serrata* or *Caridina henriettae*), and potentially undescribed species (one taxon, *i.e.*, *Halocaridinides sp.*). In marine environments, eDNA studies have sometimes detected fewer crustacean taxa than traditional sampling methods, likely due to low shedding rates and patchy distributions (Ip *et al.*, 2024). Our study demonstrates the utility of eDNA for detecting a broad range of freshwater decapod taxa, including cryptic or difficult-to-sample species. This may be attributed to the amphidromous life cycles of many species on Réunion Island, which increase the likelihood of local DNA accumulation (Keith, 2002). Moreover, the detection of likely cryptic or undescribed taxa highlights a known advantage of eDNA in revealing hidden biodiversity, particularly for groups with morphologically similar life stages or low detectability using traditional methods (Bernardes *et al.*, 2017; Siriwut *et al.*, 2021).

At the same time, the study highlights important challenges related to species detectability, database completeness, and the interpretation of community structure across spatial gradients.

4.1 Taxonomic discoveries and detection challenges

The taxonomic inventory aligns with historical knowledge of the island's decapod fauna (Keith, 2002; OCEA Consult' *et al.*, 2023), but also reveals significant novelties. The detection of *Atyoida* cf. *serrata* represents a new record for Réunion Island, and its morphological and genetic distinctiveness suggests that further taxonomic revision is needed. Similarly, the identification of *Caridina henriettae*, previously grouped under *C. serratirostris* (De Mazancourt *et al.*, 2025), reflects recent advances in resolving cryptic diversity through molecular tools. The most striking is the detection of a cave-dwelling species, *Halocaridinides sp.*, in two separate watersheds. This genus, typically associated with anchialine environments (von Rintelen *et al.*, 2012), had only recently been reported on the island (de Mazancourt *et al.*, *In press*). Its detection in river samples suggests the opportunistic use of microhabitats such as cracks or interstitials in the lower reaches of rivers by this species, but also underscores the unique capacity of eDNA to detect elusive or subterranean taxa not typically captured through traditional sampling.

The detection of marine and terrestrial taxa at river stations, such as *Geograpsus grayi* and *Cardisoma carnifex*, demonstrates the capacity of eDNA to capture non-target or

transient signals, particularly near estuarine or disturbed sites (Sahu *et al.*, 2025). While these detections are ecologically plausible given the proximity of sampling stations to the coastline or riparian environments, they also highlight the need for cautious ecological interpretation of eDNA data. Importantly, eDNA results should always be interpreted in the context of the sampling design, including the spatial distribution of sites and timing of sample collection, to avoid over- or misinterpretation of species presence.

Despite these achievements, several expected species such as *Macrobrachium lepidactylus*, *Caridina natalensis* (De Man, 1908), and the invasive *Neocaridina davidi* were not detected (Keith and Vigneux, 2000; de Mazancourt *et al.*, 2023; Prati *et al.*, 2024). Several explanations may account for these absences. First, these species may not have been present in detectable quantities at the time of sampling, reflecting natural temporal variability or low population densities (UICN France *et al.*, 2013; Dunn *et al.*, 2017). Second, they may have been present, but their DNA was either too degraded, too dilute, or not effectively amplified due to primer bias (Beng and Corlett, 2020). Third, hydrological factors such as strong stream flow, limited connectivity to suitable habitats, may have reduced local eDNA concentration below detectable levels at the time of sampling (Song *et al.*, 2017).

Comparable uncertainty surrounds taxa detected at low frequencies, such as *Varuna litterata*, likely due to unsuitable habitat conditions. This species is typically associated with estuarine and brackish environments, and our detection results at the Cimetière Marin station confirm its presence in the lower reaches of rivers on Réunion Island, particularly along the drier west coast, where freshwater input is sporadic and estuaries frequently dry out (Poupin, 2010). A similar case applies to *Macrobrachium lar*, which was detected only once in the entire dataset. This absence aligns with results from conventional monitoring programs conducted under European water quality assessment protocols, which have also reported its rarity in recent years (OCEA Consult' *et al.*, 2023). *M. lar* is known to be sensitive to water quality degradation, particularly in terms of pollution and habitat alteration (Lal *et al.*, 2014), and is additionally threatened by poaching on the island (Comité de l'eau et de la biodiversité de La Réunion, 2022).

Such findings illustrate the ecological specificity of certain species and reinforce the importance of considering habitat context in interpreting eDNA data. Overall, these observations underscore that eDNA surveys must be conducted with methodological rigor, repeated across space and time. Interpretation should also account for ecological and technical constraints, including detection thresholds, species-specific traits, and reference database completeness. Future surveys would benefit from the inclusion of positive or mock community controls to better assess detection sensitivity for rare taxa.

4.2 The case of *Macrobrachium hirtimanus*: evidence for local extinction

Among all taxa surveyed, the absence of *Macrobrachium hirtimanus* is the most ecologically and conservation-relevant finding of this study. Once widely distributed across Réunion's rivers, including the Rivière des Marsouins, Rivière Langevin, and Rivière du Mât (Kiener, 1981; Keith and Vigneux, 2000), this Mascarene-endemic species has not been confirmed since the 1980s and is listed as possibly extinct (Keith and Vigneux, 2000; UICN France *et al.*, 2013). All three basins were included in the present eDNA survey and represented priority locations for testing the persistence of the species. These rivers were selected because they historically provided suitable habitat conditions, combining permanent flow, moderate altitude gradients, and connectivity with the sea, which are essential for amphidromous prawns. Given these characteristics, if remnant populations of *M. hirtimanus* persisted anywhere on the island, eDNA traces would most likely have been detected at these sites.

Given the method's demonstrated capacity to detect both abundant and rare decapods, it is unlikely that the species persists undetected at moderate densities. While eDNA detection is probabilistic and influenced by environmental factors (Barnes and Turner, 2016), the spatial coverage and replication in this survey minimize the likelihood of false negatives.

Several ecological and anthropogenic factors may explain the disappearance of *M. hirtimanus*. First, competition and niche overlap with the morphologically similar *M. lepidactylus* may have led to competitive exclusion, as previously hypothesized by Keith and Vigneux (2000). Both species share amphidromous life histories and likely overlap in habitat preferences, but *M. lepidactylus* may be more tolerant to environmental disturbance. Second, loss of longitudinal connectivity through dams, weirs, and estuarine alterations has likely restricted larval migration between marine and freshwater habitats (Jarvis and Closs, 2019; Williams-Subiza and Epele, 2021). Given that *M. hirtimanus* requires oceanic larval development, any disruption to recruitment corridors could have resulted in reproductive failure. Third, declining water quality and habitat modification in lowland streams have further degraded suitable habitat. Agricultural runoff, sedimentation, and urbanization have altered stream substrate and vegetation cover, conditions essential for *Macrobrachium* juveniles (De Grave, 2013). Finally, demographic stochasticity and isolation effects may have amplified extinction risk. As an endemic species restricted to the Mascarenes, *M. hirtimanus* likely had small, genetically structured populations vulnerable to local extirpations and inbreeding depression (Jaisankar, 2018).

Taken together, the eDNA evidence and known ecological pressures strongly support the conclusion that *M. hirtimanus* is potentially extinct on Réunion. However, as a precautionary measure, targeted eDNA sampling in historical habitats, particularly during peak recruitment periods and in brackish transition zones, should be pursued to definitively confirm extinction. This approach could be combined with population genomic studies in Mauritius to evaluate whether conservation translocations or ex-situ preservation remain feasible options.

4.3 Alpha diversity and local environmental drivers

Community diversity analyses revealed no significant differences in alpha diversity across sites, suggesting a relatively uniform richness of detectable crustacean taxa, at the species level, throughout the study area. The identical test

outcomes for Shannon diversity and Observed richness at this level indicate a strong coupling between richness and evenness patterns, may result from the dominance of widespread amphidromous species, such as *Atyoida serrata*, *Caridina typus*, and *Macrobrachium australe*, which exhibit broad ecological tolerance (Bernardes *et al.*, 2017; Kobayashi *et al.*, 2024) and are well represented across the island (Hoarau *et al.*, 2019; OCEA Consult' *et al.*, 2023). Nevertheless, the slight divergence observed in OTU-level metrics, although not statistically significant, may point to underlying genetic or cryptic diversity not captured by species-level classifications. The similarity of alpha diversity estimates between the two sampling campaigns suggests no detectable temporal differences in community composition within the temporal resolution of this study.

This highlights the added resolution offered by eDNA metabarcoding in detecting finescale community variation, potentially associated with microhabitat differences or population structure (Jannel *et al.*, 2024). Indeed, sites such as Rivière des Roches, Rivière Sainte-Marie and Rivière Saint-Denis showed high richness, the low values observed in places such as Rivière des Marsouins upstream (MAR_2) and Petite Ravine are consistent with historical data and environmental characteristics (FDAAPPMA La Réunion, 2021; OCEA Consult' *et al.*, 2023). Indeed, the banks of these sites are rarely colonized by riparian plants, which generally provide habitat for decapod crustaceans (Torres *et al.*, 2018).

4.4 Spatial turnover and connectivity constraints

The pronounced spatial structuring of crustacean communities across Réunion's rivers, as shown by significant beta diversity and high species turnover, indicates limited overlap in species composition among sites. Beta diversity partitioning further revealed that most of the dissimilarity among sites was attributable to species turnover ($\beta_JTU \approx 0.787$), with a smaller contribution from nestedness ($\beta_JNE \approx 0.155$). This pattern suggests that community differentiation primarily results from species replacement among rivers rather than a simple loss of taxa along environmental gradients.

Interestingly, this strong turnover emerges despite the amphidromous life cycle of island decapods, which involves larval development in marine waters and subsequent recruitment into freshwater streams (Lord *et al.*, 2012). In theory, such a life history strategy should promote high dispersal potential and genetic mixing among basins (McDowall, 2010; de Mazancourt *et al.*, 2021). However, the observed structuring suggests that dispersal and recruitment are not uniform across the island. Potential drivers include oceanic currents influencing larval transport, behavioral constraints during recruitment, and anthropogenic barriers to upstream migration such as dams, altered estuaries, or unsuitable hydrological conditions (Lagarde *et al.*, 2021). The significant compositional differences between northern, southern, and eastern river communities may also reflect a combination of hydrological isolation, environmental filtering, and spatially structured recruitment dynamics. These findings highlight that even amphidromous taxa can exhibit strong spatial turnover when recruitment pathways are spatially structured or disrupted (Cook *et al.*, 2009; McDowall, 2010). Nevertheless, while eDNA provides a powerful tool for detecting aquatic taxa, detection probabilities may vary among species and environments, meaning that low-abundance or highly localized species could remain undetected. Consequently, the observed turnover may slightly underestimate true diversity patterns. From a conservation perspective, this reinforces the need to preserve multiple watershed and estuaries outlets to maintain functional connectivity and the full spectrum of crustacean diversity on the island (Keith, 2003; de Mazancourt *et al.*, 2021). For future recovery efforts of *M. hirtimanus*, this implies that even if remnant populations were discovered, their long-term persistence would depend on restoring functional river–sea corridors.

5 Conclusion

This study underscores the potential of eDNA metabarcoding for freshwater biodiversity assessment, particularly in tropical island ecosystems. It successfully identified cryptic and elusive taxa and offered insights into local- and island-scale diversity patterns. However, methodological limitations, especially those linked to incomplete reference libraries and hydrological variability, must be addressed through ongoing refinement and integration with traditional survey methods.

A key outcome of this study is the absence of any genetic signal of *Macrobrachium hirtimanus*, despite targeted sampling in historical habitats. Given the demonstrated detection capacity of the method for both common and rare taxa, this result provides strong molecular support for the hypothesis of local extinction. Nevertheless, as eDNA detection is inherently probabilistic and can be influenced by environmental and temporal factors, follow-up investigations remain essential. These should include repeated surveys over several years and expanded sampling to estuarine transition zones, using both molecular and classical field methods.

Beyond this focal species, the study also revealed several operational taxonomic units (OTUs) within Macrobrachium that could not be confidently assigned to species, underlining the urgent need to expand genetic reference databases and conduct detailed taxonomic validation to improve molecular resolution and facilitate accurate biodiversity assessments.

From a broader perspective, this research provides a critical baseline for long-term monitoring and conservation planning. The combination of new taxonomic discoveries, the absence of historically reported species such as *M. hirtimanus*, and the marked community differentiation among rivers collectively emphasize the value of eDNA metabarcoding in conservation diagnostics.

At the regional level, the status of *M. hirtimanus* should now be reassessed in accordance with IUCN criteria, which require exhaustive and repeated surveys across its historical range. Confirming its extinction status will necessitate coordinated investigations in both Réunion and Mauritius over the next 3–5 yr, combining eDNA and traditional approaches. Such work will not only clarify the fate of this emblematic Mascarene species but will also strengthen conservation frameworks for freshwater biodiversity across the region.

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

Data availability statement

NGS raw data 16SrRNA sequences are deposited in zenodo data bank: https://doi.org/10.5281/zenodo.17636668.

Author contribution statement

P.V., J.L.A, V.M. conceived the study; J.L.A., P.V., V.M. performed field work. J.L.A. performed the laboratory work. J.L.A., V.M. completed sample processing and analysis. J.L.A., V.M., P.K., P.V., P.J. contributed to final data analysis and manuscript writing.

Supplementary material

Figure S1. Workflow summary of eDNA sample processing and analytical steps. Schematic representation of the workflow from field sampling to the final analytical dataset. Three field replicates were collected at each station and processed independently through extraction, amplification, sequencing, and bioinformatics. Replicates were pooled at the statistical stage to represent one composite sample per station.

Figure S2. Rarefaction curves of the crustacean community. Each line corresponds to a sampling station.

Figure S3. Sample-size-based and coverage-based rarefaction (solid line segment) and extrapolation (dotted line segments) sampling curves with 95% confidence intervals for the eDNA dataset of Réunion island by diversity order: q = 0 (species richness).

Table S1. Summary of OTU assignments across major taxonomic groups (prior to cleaning and filtering steps), showing the number and percentage of OTUs retained for Decapoda versus non-target sequences.

The Supplementary Material is available at https://www.kmae-journal.org/10.1051/kmae/2025035/olm.

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