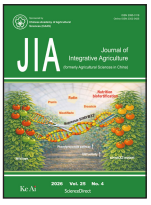
Journal of Integrative Agriculture 2026, 25(4): 1586–1596

Available online at ScienceDirect

Journal of Integrative Agriculture



RESEARCH ARTICLE

Monitoring agricultural arthropod diversity by eDNA metabarcoding from plant cleaning fluid

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Highlights

- The COI primer (mlCOIintF/jgHCO2198R) detected more rice field arthropod species compared to 16S and 18S primers in eDNA metabarcoding.
- eDNA collected from rice plant cleaning fluid (RPCF) identified 15% more arthropod species than vacuum-suction sampling.
- RPCF revealed comparable alpha diversity and taxonomic composition of arthropods between Bt and non-Bt rice fields, demonstrating its potential for monitoring agricultural arthropod communities.

Abstract

Arthropods serve essential roles in crop production as pollinators, predators, and pests. Understanding arthropod biodiversity is crucial for assessing agroecosystem health, functions, and services. Traditional survey methods are labor-intensive, costly, and rely on diminishing taxonomic expertise, limiting their agricultural applications. Environmental DNA (eDNA) metabarcoding of diverse samples provides comprehensive species composition data through efficient and non-invasive sampling. However, this method remains underutilized in rice field studies. This research examined four sample substrates — rice plant cleaning fluid (RPCF), rice pollen, soil, and water — using various barcoding primers to identify optimal substrates for monitoring rice paddy arthropod diversity. The method was implemented in Bt rice and non-Bt rice fields to evaluate its biomonitoring potential. Results indicate that the COI primer (mlCOIintF/jgHCO2198R) identified the highest number of rice field arthropod species. The eDNA collected from RPCF detected 15% more arthropod species compared to vacuum sampling of whole arthropods. Rice pollen collection during the heading stage also revealed considerable arthropod diversity. Alpha diversity and taxonomic composition remained consistent between Bt and non-Bt rice fields, aligning with traditional survey findings. These results suggest that eDNA metabarcoding of plant cleaning fluid offers an effective approach for monitoring agricultural arthropod communities, contributing to agricultural production optimization.

Keywords: arthropod diversity, environmental DNA, agroecosystem, Bt rice

1. Introduction

Plant and arthropod species maintain essential relationships in agroecosystems, encompassing herbivory, predation, parasitism, and pollination (Zhu *et al.* 2015; Dunn *et al.* 2018). Rice paddy ecosystems support diverse taxonomic and functional arthropod groups, including phytophagous insects, pollinators, predators, parasites, and other taxa. A diverse and stable rice paddy arthropod community enhances biological control through natural enemies of pests, potentially reducing pest damage and increasing rice yield (Gurr *et al.* 2016). Despite their critical role in agroecosystem functioning, arthropod species, along with animal species broadly, are experiencing significant declines (Dirzo *et al.* 2014; Finn *et al.* 2023). The decline in arthropod biodiversity

Received 5 February 2025; Received in revised form 28 April 2025; Accepted 3 June 2025; Available online 14 July 2025
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doi: 10.1016/j.jia.2025.07.014

is significant, as it can diminish plant reproductive success, accelerate pest spread, and cause economic losses (Smith *et al.* 2015; Reilly *et al.* 2020). This challenge will likely intensify with increasing pressures on ecological systems due to resource utilization and climate change. The examination of arthropod diversity in rice cropping systems remains essential for enhancing rice sustainability and productivity.

Monitoring arthropod diversity is fundamental to assessing and managing rice ecosystem health (Letourneau *et al.* 2011; Senapathi *et al.* 2021; Kestel *et al.* 2022). Traditional identification of these taxa has primarily depended upon passive trapping (e.g., pan, Malaise, and surface cup traps), visual observation, and active survey techniques (e.g., vacuuming, basin-beating, sweep netting; (Spafford and Lortie 2013; Prado *et al.* 2017; Gervais *et al.* 2018; McCravy 2018). Malaise trapping, basin-beating, and vacuuming represent common methods for evaluating arthropod diversity in rice paddy and other ecosystems. However, these methods typically require substantial time investments and increasingly scarce taxonomic expertise to identify arthropod taxa in field collections (Brown 2021). When morphological identifications are feasible, these sampling techniques often exhibit inherent biases in captured taxa. For instance, Malaise traps (René Malaise described the trap in 1934) capture various flying insects, but exclude non-flying arthropods. The basin-beating method captures more arthropods at the plant base, but primarily predatory taxa (Wang *et al.* 2016), limiting comprehensive determination of arthropod communities in rice cropping systems. Although vacuum sampling proves most efficient among the three methods, it risks habitat destruction (Wang *et al.* 2016). The development of new, non-destructive survey approaches that offer greater efficiency and cost-effectiveness than conventional methods appears necessary.

Environmental DNA (eDNA) collection and metabarcoding has emerged as a novel approach in ecological research (Thomsen and Willerslev 2015; Wilson *et al.* 2019; Orr *et al.* 2021). This noninvasive analysis method has demonstrated effectiveness in monitoring biodiversity across marine, lotic, lentic, and terrestrial ecosystems (Yamamoto *et al.* 2017; Djurhuus *et al.* 2020; Valentin *et al.* 2020; Visser *et al.* 2021). eDNA analysis shows promise as a complement to conventional sampling methodologies. In agroecosystems, arthropod eDNA research has progressed along several lines of investigation. Studies have utilized eDNA from wildflowers (Thomsen and Sigsgaard 2019) and pollen attached to arthropods to examine plant-pollinator interactions (Thomsen and Sigsgaard 2019; Bell *et al.* 2022; Johnson *et al.* 2023; Kestel *et al.* 2023). Additionally, eDNA analyses of topsoil enable monitoring of soil arthropod communities (Zinger *et al.* 2019; Arribas *et al.* 2021). For plants, eDNA collected from leaf material (Valentin *et al.* 2020; Allen *et al.* 2021) and freshwater (Tréguier *et al.* 2014; Brown *et al.* 2016) has enabled rapid and efficient identification of invasive species. However, eDNA metabarcoding studies in rice paddy ecosystems remain unreported. Notably, arthropod eDNA sampling in terrestrial ecosystems presents challenges due to the diverse habitats these arthropods occupy. Limited studies sampling multiple terrestrial substrates reveal substantial variation in community composition across substrates, highlighting the importance of appropriate metabarcoding sample selection for terrestrial surveys (van der Heyde *et al.* 2020).

Despite the rapid advancement of eDNA analysis, its full scope of application remains to be determined. This study hypothesizes that systematic eDNA-driven surveys of specialized arthropod communities in rice paddies can provide valuable insights into community structures and population dynamics. The method was applied to Bt rice and non-Bt rice fields to evaluate its potential for biomonitoring. This paper presents the results of experiments designed to test this hypothesis.

2. Materials and methods

2.1. Field site

The experiments were conducted at the Changxing Agricultural Experiment Station of Zhejiang University, China (30°30′N, 119°24′E). The study utilized an insect-resistant transgenic rice line expressing *Cry2A* gene derived from Bt and its near isogenic control line MH63. The Bt rice (T2A-1) demonstrated high resistance to rice stem borer *Chilo suppressalis*, and yellow stem borer, *Scirpophaga incertulas* (Chen *et al.* 2008). T2A-1 and non-Bt rice (MH63) were cultivated in two fields separated by 15 m (Appendix A). Four eDNA substrates — rice plant cleaning fluid (RPCF), rice pollen, rice fields soil, and water — were collected during four rice developmental stages: tillering, jointing, heading, and post-harvest, during 2021 and 2023. Concurrent vacuum-suction sampling (Carino *et al.* 1979) was employed to collect arthropods. To ensure unbiased sampling between methods, three eDNA sampling plots and three suction sampling plots were established for each rice variety. Each plot measured 20 m×20 m, with 1.5 m spacing between plots. Rice seeds were sown in June 2021 and June 2023. One month after seeding, rice plants were transplanted as single seedlings, spaced 16.5 cm apart. No pesticides were applied during the growing season, while fertilizer and irrigation management followed local farming practices.

2.2. Arthropod sampling

Arthropod eDNA extraction from RPCF, rice pollen, soil, and water was conducted using the following procedures. For surface sampling of rice plants, plants were harvested at root level using a sickle and transported to the laboratory, where they were cleaned with 1,000 mL of deionized water, which was subsequently collected. Five rice plants were randomly selected from each plot, totaling 15 rice plants per sampling method for each rice variety. Rice pollen collection occurred during the heading stage. Rice spikes were gently tapped to collect fallen pollen on white porcelain discs (36 cm× 46 cm×3.5 cm). The pollen (0.5 g/plot) was transferred to 1.5 mL centrifuge tubes. Within each plot, five soil samples were collected randomly using a 60 mL ring cutter and stored in zip-lock bags, with three samples total per rice variety. Following collection, samples were cleaned of large stones and leaves, then frozen at –20°C for subsequent molecular analysis of arthropod diversity. Water samples from rice paddy fields were collected five times using a 50 mL

centrifuge tube and combined in a beaker, measuring 250 mL per plot and 1,500 mL total for each rice variety.

For suction sampling, arthropods were collected using a vacuum-suction machine (model 1612, John W. Hock Company, Gainesville, Florida, USA) within a three-dimensional sampling frame (50 cm length by 50 cm width by 90 cm height; plane area: 0.25 m^2; made of Mylar sheets; Carino *et al.* 1979). The sampling frame was positioned randomly along plot diagonals, with five samples taken per plot. Each sampling event lasted approximately 2 min. Arthropods were collected in nylon sock collecting bags, subsequently isolated under a binocular microscope (Olympus SZX7), and preserved in 100% ethanol prior to DNA extraction. Four eDNA samples and suction sampling were performed during the heading stage of MH63 in 2021, while RPCF and suction sampling were employed during other rice stages.

2.3. DNA extraction and PCR amplification

RPCF and water samples were filtered through a microporous filter membrane (0.45 μmol L^{-1} pore size). The membrane was then sectioned for DNA extraction using the HiPure Water DNA Kit (Magen, Shanghai, China). Soil sample DNA was extracted using the HiPure Soil DNA Kit (Magen), while rice pollen and suction samples underwent DNA extraction using the HiPure Universal DNA Kit (Magen). All DNA extractions followed the manufacturer's prescribed protocols.

The study employed two pairs of universal cytochrome *c* oxidase I (COI) primers (mlCOIintF/jgHCO2198R; ZBJ_Art_F/ZBJ_Art_R) (Zeale *et al.* 2011; Leray *et al.* 2013), 16S primers 16S_216_F and 16S_216_R (Elbrecht *et al.* 2016), and 18S primers 18S_110_F and 18S_110_R (Zinger *et al.* 2019) (Table 1). These four primer pairs were utilized for simultaneous PCR DNA amplification of suction samples collected during the 2021 rice heading stage, while COI primers were applied to all other stage samples. PCR reactions were conducted in 20 μL volumes containing 4 μL of 5×FastPfu buffer, 2 μL of dNTPs, 0.8 μL (5 μmol L^{-1}) of each forward and reverse primer, 0.4 μL of FastPfu polymerase, 10 ng of DNA template, with double-distilled H_2O (ddH_2O) added to achieve final volume. The amplification protocol began with initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, primer-specific annealing for 30 s (mlCOIintF/jgHCO2198R: 62°C; ZBJ_Art_F/ZBJ_Art_R: 53°C; 16S_216_F/16S_216_R: 52.5°C; 18S_216_F/18S_216_R: 45°C), and extension for 45 s at 72°C, concluding with a 10 min extension at 72°C. Three PCR replicates were performed per sample, combined, thoroughly mixed, and verified on 2% Agarose gels. Mixed PCR products were purified using the AxyPrepDNA Gel Recovery Kit (AXYGEN Biotech, Shanghai, China) following manufacturer specifications.

2.4. DNA library preparation and sequencing

Purified PCR products were quantified using Qubit®3.0 (Thermo Fisher Scientific, Waltham, MA, USA), and every twenty-four amplicons with different barcodes were mixed in equal proportions. The pooled DNA product was utilized to construct an Illumina Pair-End library following Illumina's genomic DNA library preparation procedure. The amplicon library underwent paired-end sequencing (2×250) on an Illumina MiSeq platform (Shanghai BIOZERON Co., Ltd., China) according to standard protocols.

2.5. Data processing

Sample data were distinguished by index sequence and stored in fastq format. Quality control was conducted on the reads using FASTQC (Andrews 2010), with bases below quality value 20 at the tail being trimmed using a 50 bp window. When average quality within the window fell below 20, bases from the window's start were truncated. Reads shorter than 50 bp post-quality control were removed. Paired-end (PE) reads were merged into sequences with a minimum 10 bp overlap. The maximum permitted error rate in the overlap region was 0.2%, and non-matching sequences were excluded. Samples were distinguished by barcodes and primers, with sequence orientation adjusted; barcode mismatches were not permitted, while up to two primer mismatches were allowed. The filtered reads were then demultiplexed using OBITOOLS (Boyer *et al.* 2015), and sequences shorter than 120 bp were removed. Chimeras were eliminated using Usearch (Edgar 2016) through combined *denovo* and reference methods. Operational taxonomic units (OTUs) were clustered using Usearch (Edgar 2016): non-repetitive sequences were extracted to reduce computational redundancy, single sequences without repetitions were removed, and remaining non-repetitive sequences were clustered at 97% similarity to form OTUs. Chimeras were removed during clustering to yield representative OTU sequences. All optimized sequences

Table 1 The sequences of four universal primers used in this study

Primers	Sequence	References
COI		
mlCOIintF	5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′	Leray *et al.* (2013)
jgHCO2198R	5′-TAAACTTCAGGGTGACCAAARAAYCA-3′	Leray *et al.* (2013)
ZBJ_Art_F	5′-AGATATTGGAACWTTATATTTTATTTTTGG-3′	Zeale *et al.* (2011)
ZBJ_Art_R	5′-WACTAATCAATTWCCAAATCCTCC-3′	Zeale *et al.* (2011)
16S		
16S_216_F	5′-TRRGACGAGAAGACCCTATA-3′	Elbrecht *et al.* (2016)
16S_216_R	5′-TCTTAATCCAACATCGAGGTC-3′	Elbrecht *et al.* (2016)
18S		
18S_110_F	5′-TTTGTCTGSTTAATTSCG-3′	Zinger *et al.* (2019)
18S_110_R	5′-TCACAGACCTGTTATTGC-3′	Zinger *et al.* (2019)

were mapped to these representative sequences, selecting those with above 97% similarity to generate the OTU table.

OTU identification was performed through BLASTN searches (Altschul *et al.* 1990) of representative sequences against the NCBI nucleotide database (ftp://ftp.ncbi.nlm.nih.gov/; accessed 30/6/2021) and the Barcode of Life Data System (BOLD; http://www.boldsystems.org/index.php; accessed 12/6/2021). For suction samples collected during the 2021 rice heading stage, two databases were employed for species annotation, while samples from other stages primarily utilized NT database results with reference to BOLD database findings. A minimum hit length of 90% of the query sequence was required. Species-level assignments required ≥99% similarity, genus-level assignments ≥95% similarity, and higher-level assignments ≥92% similarity. For this study, arthropod data were filtered and unreasonable species manually removed. To minimize taxonomic errors from public databases, assignments for groups with known database issues were cross-verified between NCBI and BOLD. Discrepant records were downgraded to genus-level or excluded. All species annotations were evaluated against previous monitoring records (Lu *et al.* 2016) to exclude biogeographically implausible records. The annotated OTU species data underwent statistical analysis, and community composition was assessed at various taxonomic levels: class, order, family, genus, and species.

2.6. Statistical analyses

Statistical analyses were conducted using R version 4.3.0 (R Core Team 2023). Arthropod catches from three plots were combined to provide field-level arthropod data. Alpha diversity comparisons at the arthropod species level among five sampling methods were performed using one-way ANOVA and Tukey's HSD posthoc analysis (Ma *et al.* 2023). Shannon alpha diversity indices at both arthropod family and species levels were compared between rice varieties using the non-parametric Kruskal-Wallis test. Arthropod family assemblage dissimilarities within sampling methods and rice varieties were examined using permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distances and 9999 permutations (Anderson 2017). Additionally, principal coordinate analysis (PCoA) with Bray-Curtis distances was performed. These analyses utilized the "vegan" package (Oksanen *et al.* 2022).

3. Results

3.1. Primers and databases comparison

Simultaneous PCR amplification using four universal primer pairs was performed on DNA from samples collected *via* suction sampling during the heading stage of MH63 rice. Among the four primer pairs, the mlCOIintF/jgHCO2198R primers generated 865 OTUs and annotated the most rice paddy arthropod species, representing 4 identifiable arthropod classes: Arachnida, Insecta, Branchiopoda, and other arthropods (Table 2). Based on these results, this universal primer was selected for subsequent eDNA metabarcoding sequencing from rice fields.

The high-throughput sequencing results using COI primers were annotated utilizing the Nucleotide Sequence Database (NT database) and the arthropod database in BOLD. The NT database yielded a higher number of annotated species compared to the arthropod barcode database across all taxonomic levels: phylum, class, order, family, genus, and species (Table 3). Among 865 OTUs, 323 showed divergent annotation results between the databases. Analysis of these inconsistent OTUs revealed that the NT database provided more precise species annotations. For instance, 56 OTUs were identified as *Cyrtorhinus lividipennis*in the NT database but only as Miridae in the arthropod barcoding database. Similarly, 29 OTUs were classified as *Bryophaenocladius* in the NT database but broadly as Diptera in the arthropod barcode database, while 11 OTUs identified as *Megaselia* in the NT database were classified only as Phoridae in the arthropod barcode database. Twenty-seven OTUs were identified as non-arthropods in the NT database. Subsequent analyses primarily utilized the NT database annotations while considering the arthropod barcode database results as reference.

Table 2 DNA metabarcoding results from rice paddy arthropods using four universal primers

Primers	PCR length (bp)	Cleaned reads	OTUs	Classes	Orders	Families	Species
mlCOIintF/jgHCO2198R	313	1,283,917	865	4	13	65	112
ZBJ_Art_F/R	157	1,371,242	1,281	2	6	39	109
16S rRNA F/R	214	1,299,323	311	1	4	21	49
18S rRNA F/R	150	1,236,288	255	3	8	22	34

Table 3 Comparison of arthropod taxonomic annotation results between two databases using COI primers

Taxonomic level	Annotation number		Not consistent annotation result of OTUs between two databases at each taxonomic level
	NT database	Arthropod barcode database	
Phylum	7	1	26
Class	12	4	47
Order	24	14	71
Family	77	48	155
Genus	99	42	248
Species	119	82	323

3.2. Comparing eDNA and vacuum-suction sampling methods

A comparison of eDNA technique and suction sampling was conducted by combining results from four substrates. The eDNA metabarcoding method identified more arthropod taxa at class, order, and family levels compared to suction sampling (Fig. 1). Through eDNA analysis, 66 taxa were identified: six at class level, 14 at order level, and 46 families, with Paronellidae, Cyclopidae, and Coccinellidae showing highest abundance. Suction sampling detected three classes, six orders, and 24 families, with Coccinellidae, Delphacidae, and Paronellidae being most abundant.

Taxa composition and diversity exhibited significant variation across substrates (Fig. 2). The rice paddy soil layer revealed three classes, eight orders, and 14 families, with Paronellidae, Staphylinidae, and Linyphiidae as dominant families. The rice paddy water layer contained four classes, eight orders, and 15 families, predominantly Cyclopidae, Staphylinidae, and Paronellidae. RPCF samples demonstrated the highest arthropod diversity with four classes, 11 orders, and 28 families, dominated by Paronellidae, Coccinellidae, and Delphacidae. Rice pollen samples contained three classes, 10 orders, and 21 families, with Coccinellidae, Delphacidae, and Paronellidae as dominant families.

Among all sampling methods, eDNA collected from RPCF demonstrated the highest alpha diversity of arthropod species compared to suction sampling (Fig. 3). Arthropod species richness showed significant variation across the five sampling methods (ANOVA, $P<0.001$; Fig. 3-A), while arthropod Shannon diversity and evenness indices remained consistent (ANOVA, $P=0.36$; Fig. 3-B and C). Based on these findings, RCPF is recommended as the optimal source for arthropod eDNA sampling in paddy fields.

3.3. Arthropod diversity and composition in Bt and non-Bt rice fields

The effectiveness of the mlCOIintF/jgHCO2198R primer amplification system and RPCF sampling for monitoring arthropod diversity was evaluated by comparing eDNA samples from RPCF and suction samples collected during the heading stage of Bt rice (T2A-1) and non-Bt rice (MH63). The arthropod Shannon alpha diversity indices at both family and species levels showed no significant differences between Bt and non-Bt rice (Kruskal-Wallis test, $P=0.90$; Fig. 4-A) or between sampling methods (Kruskal-Wallis test, $P=0.20$; Fig. 4-A). Arthropod family composition demonstrated clear differentiation between sampling methods ($R^2=0.10$, $P=0.002$; Fig. 4-B), while Bt and non-Bt rice showed no significant differences ($R^2=0.06$, $P=0.143$; Fig. 4-B). The combined methods detected 287 species from 91 families within 17 orders in both rice field types, though neither method alone captured the complete arthropod diversity. Suction sampling in the Bt rice field yielded the highest number of arthropod families among the four groups (Fig. 4-C).

At the family level, eDNA collected from RPCF in Bt and non-Bt paddies identified co-abundant families including Paronellidae, Coccinellidae, Delphacidae, and Entomobryidae, while co-abundant families collected through suction sampling included Coccinellidae, Chrysomelidae, Drosophilidae, and Culicidae (Fig. 5-A). At the species level, the eDNA species from RPCF in Bt and non-Bt rice fields included *Akabosia matsudoensis*, *Nilaparvata lugens*, *Limnophyes minimus*, and *Stenus cicindeioides*, while samples obtained through suction sampling revealed abundant species including *Nilaparvata lugens*, *Akabosia matsudoensis*, and *Phaedon brassicae* (Fig. 5-B).

4. Discussion

The findings presented in this paper substantiate our

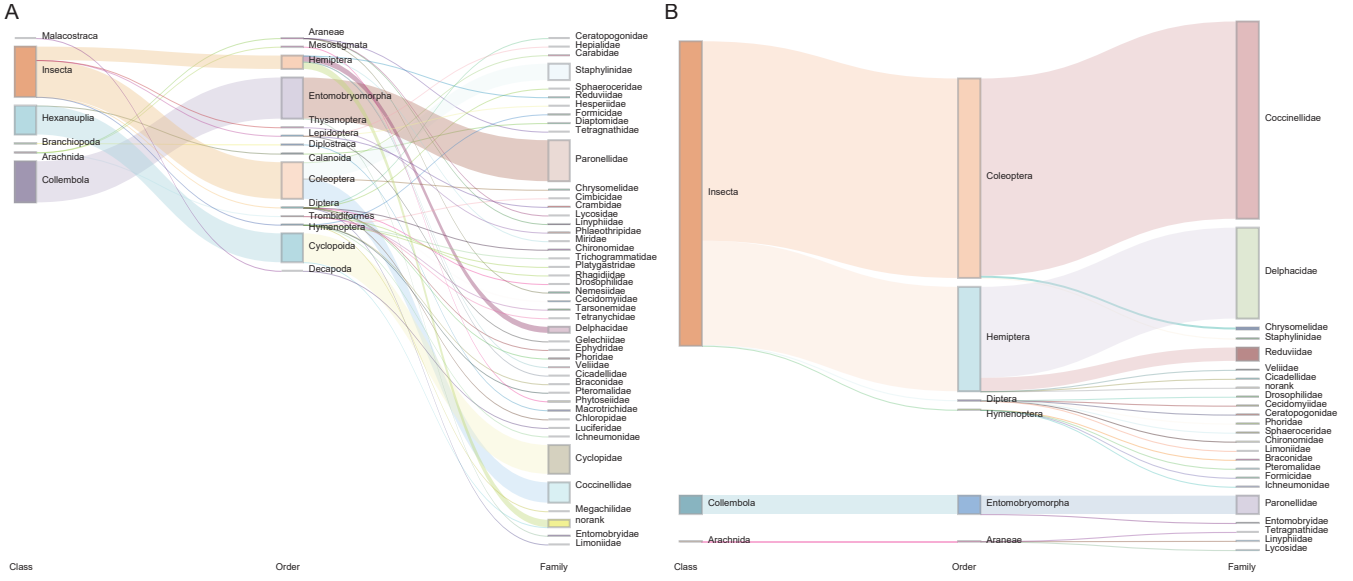


Fig. 1 The arthropod class, order, and family identified from eDNA (A) and vacuum-suction samples (B) during the heading stage of MH63 rice.

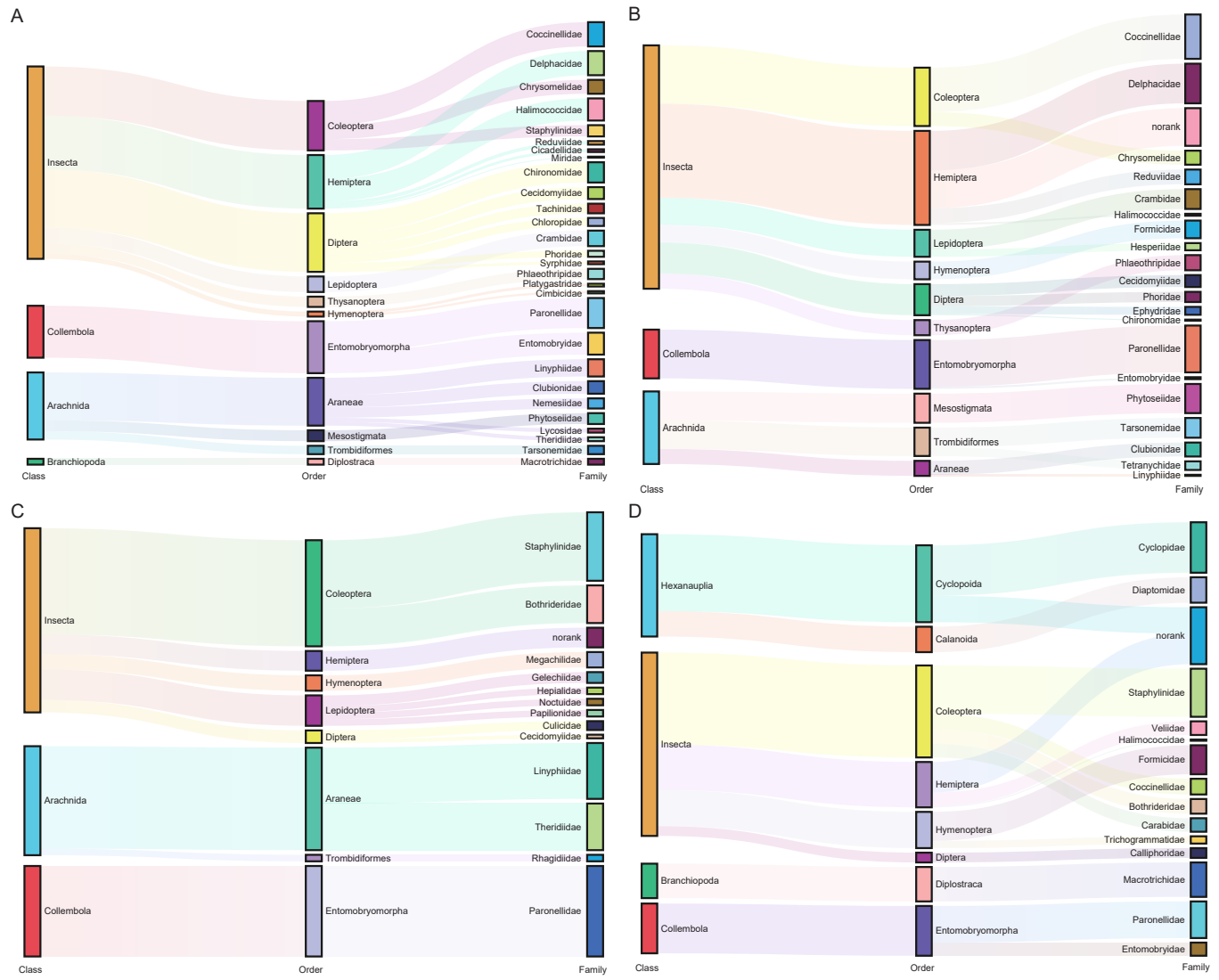


Fig. 2 Distribution of arthropod taxa in the heading stage of MH63 rice across rice plant cleaning fluid (A), rice pollen (B), rice paddy soil layer (C), and rice paddy water layer samples (D).

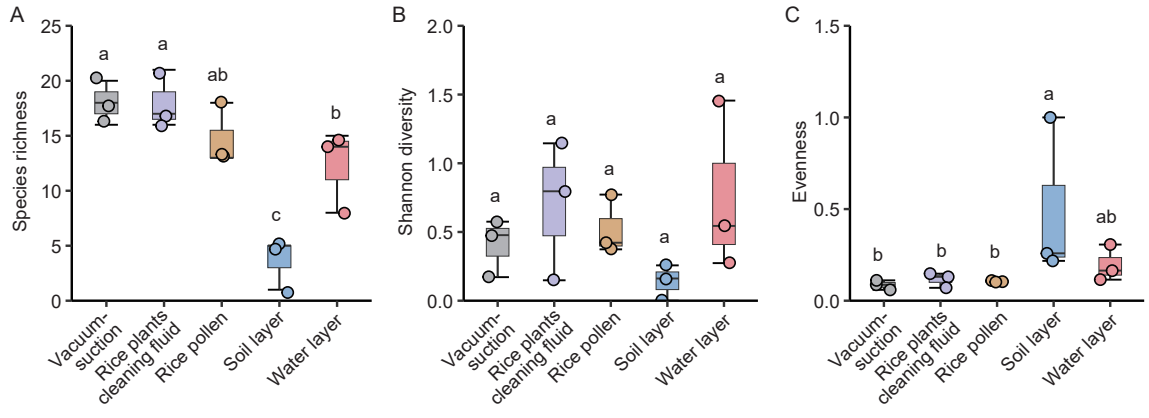


Fig. 3 Arthropod species richness (A), Shannon diversity (B), and evenness indices (C) at the species level across different eDNA samples during the heading stage of MH63 rice. Letters denote statistically significant differences between groups based on Tukey's HSD posthoc analysis (P<0.05).

hypothesis that systematic eDNA-driven surveys of specialized arthropod communities in rice paddies provide valuable insights into community structures and population dynamics. Several key observations emerge. First, the

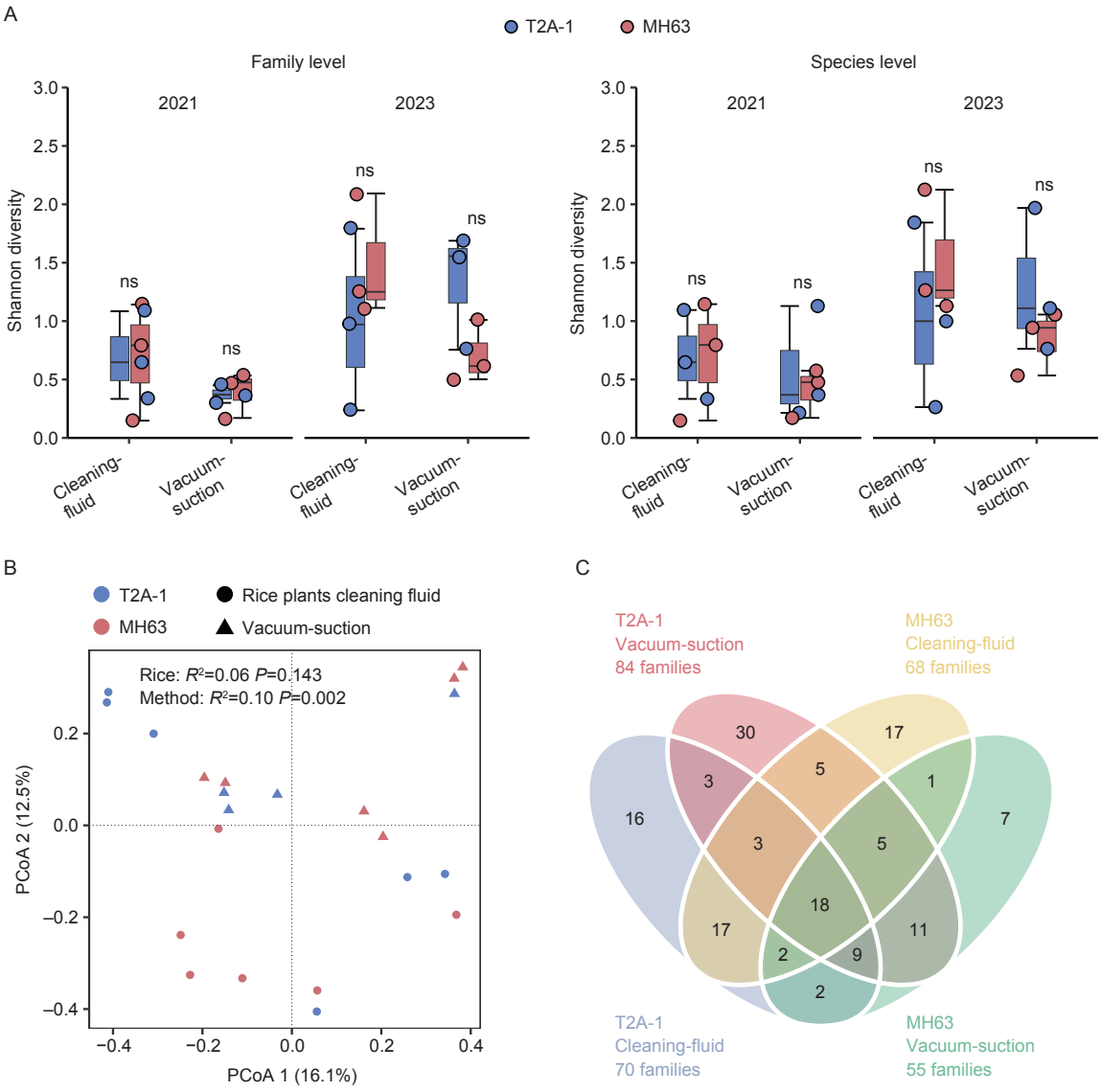


Fig. 4 Arthropod diversity and composition recorded by eDNA (RPCF) and vacuum-suction methods in Bt (T2A-1) and non-Bt (MH63) rice fields at heading stage. A, Shannon diversity for arthropod families and species. ns, no significant difference. B, principal coordinate analysis (PCoA) of arthropod family assemblage based on Bray-Curtis dissimilarity, showing relationships by survey method and rice variety. C, number of families identified for each survey method and rice variety.

COI primer, mlCOIintF/jgHCO2198R, generated the highest number of OTUs. Second, arthropod species annotation in the NT database revealed greater species coverage and accuracy than arthropod barcode database. Additionally, arthropod Shannon alpha diversity indices showed no significant differences between Bt and non-Bt rice. These findings support our hypothesis and demonstrate the effectiveness of analyzing eDNA from RPCF compared to alternative methods.

Our evaluation identified a pair of universal barcode primers suitable for PCR amplification of arthropod eDNA in rice fields. Compared to conventional survey methods, metabarcoding eDNA collected from RPCF enhanced arthropod species detection by 15%. In alignment with previous studies, eDNA metabarcoding enabled detection of potential pollinators, non-target herbivores, and natural enemies without requiring taxonomic expertise (Vorholt 2012; Gamonal Gomez *et al.* 2023; Johnson *et al.* 2023). The method's accuracy depends on arthropod eDNA quantity and quality, non-target DNA contamination, sequence analysis methodology, and availability of arthropod sequences in public databases (Ficetola *et al.* 2016; Valentin *et al.* 2021). Nevertheless, this study demonstrates eDNA sampling's potential for detecting non-target arthropod communities in Bt crops. During the rice heading stage, arthropod family and species diversity detected through eDNA showed no significant differences between Bt and non-Bt rice fields. The eDNA metabarcoding of plant cleaning fluid offers substantial potential for improving arthropod community monitoring. Generating comparable data requires standardized approaches consistently implemented in survey programs.

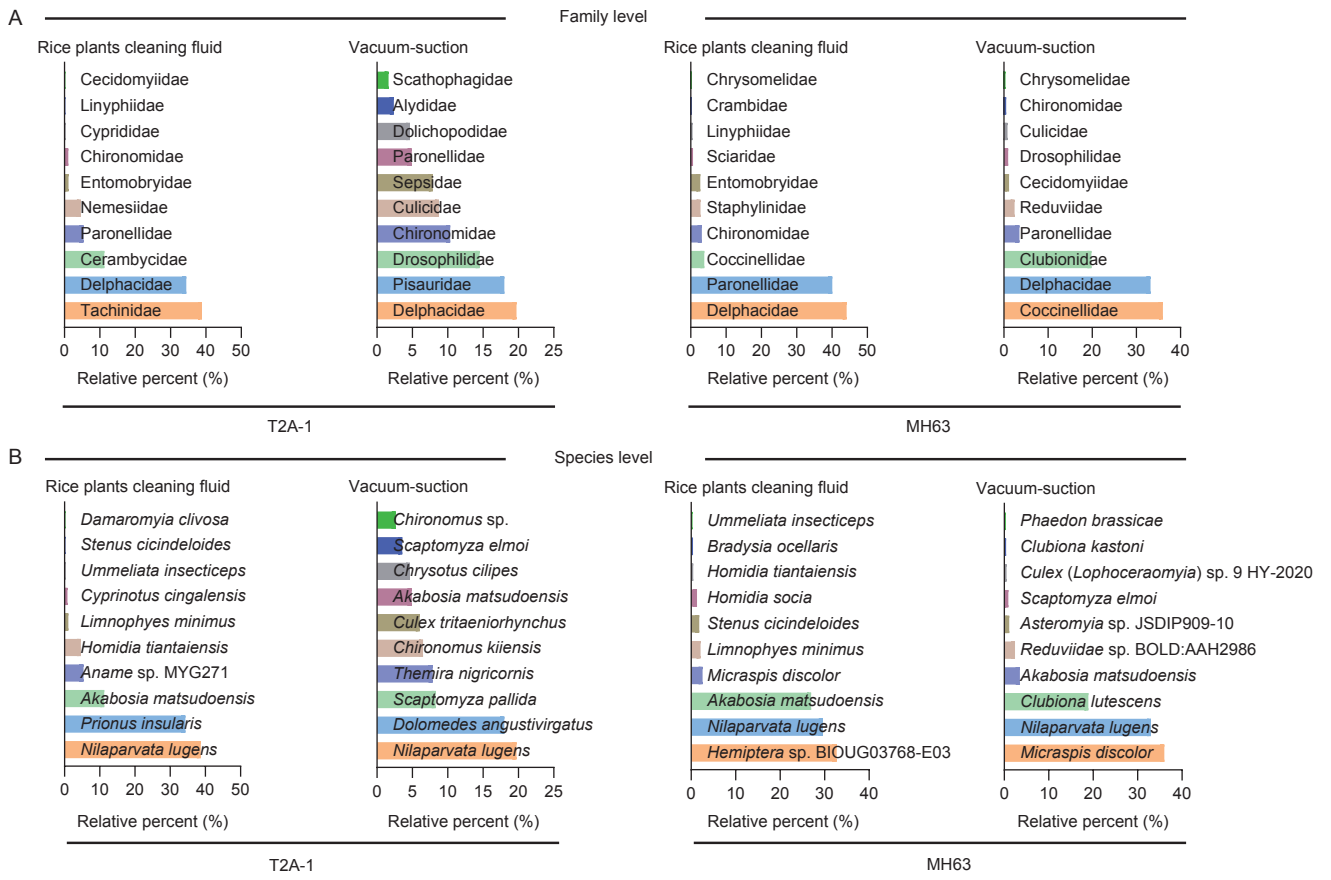


Fig. 5 Predominant families (A) and species (B) of arthropods identified through dual sampling methodologies during the heading stage of Bt- (T2A-1) and non-Bt (MH63) rice.

4.1. Primers, database and sampling considerations

The generic primers utilized in this study were designed for metabarcoding of degraded DNA and previously validated (Zeale *et al.* 2011; Leray *et al.* 2013; Elbrecht *et al.* 2016; Zinger *et al.* 2019; Zenker *et al.* 2020). Given the short amplicon size, the COI gene primer, mlCOIintF/jgHCO2198R, provided superior taxonomic coverage and resolution of arthropod communities. While the COI gene primer, LCO1490/HCO2198, is considered more suitable for arthropod classification, its amplified fragment spans 658 bp (Folmer *et al.* 1994), exceeding the maximum sequence length of approximately 500 bp in the current Illumina PE250 sequencing platform. Most studies combine the mlCOIintF forward primer with the reverse primer jgHCO2198, amplifying the 313 bp "mini-barcode region" in the 3′ of the Folmer fragment. Although eDNA metabarcoding employs universal primers theoretically providing equal amplification efficiency across species, variations in template abundance can introduce bias during PCR amplification, potentially misrepresenting actual community composition. Limited analysis might overlook ecologically significant taxa. Some eDNA metabarcoding studies indicate that additional PCR replicates on identical samples could increase recovered diversity (Valentin *et al.* 2016; Thomsen and Sigsgaard 2019). Conversely, soil fungi metabarcoding research suggests increased sequencing depth provides more reliable diversity capture than PCR replication (Smith and Peay 2014).

Previous research has examined phylogenetic relationships and species identification through arthropod sequence comparisons against GenBank and BOLD databases (Meiklejohn *et al.* 2019). This study indicates that GenBank provides more accurate genus- and species-level identifications compared to BOLD. In the database comparison, approximately 10% of OTUs demonstrated sequence similarity below 90%. The limited database coverage for arthropods presents a significant challenge for eDNA-based biodiversity studies (Li *et al.* 2022). Public barcoding databases face several constraints that affect accurate arthropod species annotation, including variable sequence coverage across barcoding *loci*, differential success rates of *loci* for specific taxon groups (e.g., 16S ineffectiveness for insect identification), taxonomic inaccuracies or gaps, and unidentified arthropod species (Saccò *et al.* 2022). Barcode data errors frequently occur due to human factors, including specimen misidentification, sample confusion, and contamination (Cheng *et al.* 2023). Species identifications in databases like GenBank warrant scrutiny due to potential inaccuracies and the absence of voucher specimens for validation. Despite cross-validation between GenBank and BOLD, this study could not entirely eliminate species barcoding errors present in public databases. For instance, sequences of common

rice hemipteran pests (e.g., *Nephotettix virescens*) proved unreliable (Cheng *et al.* 2023), necessitating the development of local rice field arthropod databases for verification. Reliable specimen proportion and identification require consistent sampling strategies to optimize arthropod species detection. Advanced classification tools can complement GenBank and BOLD to enhance confidence levels (e.g., machine learning, RDP classifier, Pro-Tax) (Abarenkov *et al.* 2018; Porter and Hajibabaei 2018). Research has demonstrated methods for locally merging sequences from both databases to maximize taxonomic coverage and reliability (Macher *et al.* 2017). Furthermore, database utility can be enhanced through custom-made or hybrid databases, though this requires additional resources depending on missing focal taxa. Comprehensive reference databases require collective responsibility, including public data sharing. This encompasses support for researchers contributing high-quality data and fostering collaborative database maintenance efforts. Additionally, local initiatives should emphasize bioinformatics education and training to address database-specific challenges.

The study employed a 97% sequence similarity threshold for OTU clustering, a standard commonly used in bacterial 16S rRNA gene analyses and COI-based arthropod studies. However, this threshold may not fully account for the varying evolutionary rates and intra/interspecific diversity among arthropod groups. For instance, certain insect orders (e.g., Diptera) show higher intraspecific COI variation (>3%) than others (e.g., Coleoptera), potentially resulting in over-splitting of conspecific sequences or under-splitting of cryptic species (Pentinsaari *et al.* 2020). This limitation could affect diversity estimates in taxa with elevated mitochondrial substitution rates or obscure ecological patterns in communities dominated by closely related species. While the 97% threshold facilitates cross-study comparisons, future methodological improvements, such as dynamic thresholding or phylogenetic information integration, may better represent arthropod diversity in agricultural settings.

Multiple rice field substrates were tested, revealing that diverse arthropod taxonomic groups can be identified across all substrates (Fig. 2). RPCF emerged as the most taxonomically diverse substrate, detecting 28 arthropod families, including parasitoids (Platygastridae, Cimbicidae), predators (*Micraspis discolor*), and pests (*Nilaparvata lugens*). Additionally, eDNA collected from RPCF demonstrated the highest consistency in arthropod diversity compared to suction samples (Fig. 3). The arthropod families strongly associated with plant samples are primarily those that feed and reproduce on living plants, providing substantial opportunity for DNA deposition (van der Heyde *et al.* 2020). Plant cleaning fluid has been demonstrated effective for broad biodiversity surveys in previous studies. Valentin *et al.* (2020) utilized spray collection and trunk roller methods to collect eDNA from plant surfaces for investigating *Lycorma delicatula* population size in forests (Valentin *et al.* 2020). Rice pollen analysis revealed high arthropod family diversity, with pollinators comprising half of the detected species. Additional studies confirm that arthropod eDNA metabarcoding on flowers enhances detection of species richness and composition (Thomsen and Sigsgaard 2019; Bell *et al.* 2022; Newton *et al.* 2023). The use of eDNA from rice pollen during the rice heading stage is recommended.

4.2. Potential applications of eDNA metabarcoding of RPCF

The research revealed no significant differences in arthropod diversity and composition between Bt and non-Bt rice using eDNA collected from RPCF (Figs. 4 and 5), aligning with numerous studies using conventional sampling methods (Dang *et al.* 2017; Li *et al.* 2017). While metabarcoding of arthropod eDNA from RPCF shows promise as a method for measuring arthropod diversity, several areas require attention for future research improvement. First, expanding eDNA metabarcoding across temporal and spatial scales while optimizing the system would enhance eDNA-based surveys through standardized sampling and sequencing procedures. Second, accessibility to eDNA data will improve as NGS costs decrease and sequencing technologies advance, enabling larger volumes of accurate DNA sequence data in shorter timeframes (Zafeiropoulos *et al.* 2020; Mousavi-Derazmahalleh *et al.* 2021). The extensive number of arthropod taxa in agroecosystems presents ongoing challenges in DNA sequence identification, necessitating arthropod taxa-specific databases (Berry *et al.* 2021). Third, pilot studies should establish detection thresholds to determine DNA deposition and degradation rates for different arthropod species and quantify amplifiable fragment size ranges (Valentin *et al.* 2021). These studies should consider local abiotic and biotic factors (e.g., UV light or microbial activity) (Strickler *et al.* 2015) to contextualize species assemblages from eDNA detections. Implementation of these recommendations may enhance the accuracy and reliability of RPCF-based arthropod eDNA metabarcoding in rice fields.

5. Conclusion

This research compared arthropod communities detected through eDNA metabarcoding of four substrates (RPCF, soil, water, and pollen) with suction sampling, a conventional survey technique. The findings demonstrate that eDNA collected from RPCF increased identified arthropod species by 15%. Rice pollen collection at the heading stage revealed significant arthropod diversity. Among different barcoding primers, the COI primer (mlCOIintF/jgHCO2198R) identified the highest number of rice field arthropod species. The study confirms that alpha diversity levels and taxonomic composition of arthropods remained consistent across Bt and non-Bt rice fields using RPCF-collected eDNA, corroborating traditional survey method results. The research establishes the necessity for standardized approaches in survey programs. These findings indicate that eDNA metabarcoding of plant cleaning fluid offers potential improvements in agricultural arthropod community monitoring, contributing to agricultural production optimization.

Acknowledgements

This work was supported by the Biological Breeding-Major

Projects of China (2023ZD04062), the China Postdoctoral Science Foundation (2021M702880), and the Fundamental Research Funds for the Central Universities, China (226-2024-00070).

Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical statements

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Appendix associated with this study is available at https://doi.org/10.1016/j.jia.2025.07.014

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