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Can eDNA Present in Aquatic Environments of Rural Areas Help Identify Species Diversity in the Order Anura?

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Abstract: Paddy fields are classified as wetland environments, and they comprise freshwater ecosystems. They are ecologically important habitats and breeding grounds for many aquatic insects, amphibians, and reptiles. However, paddy field ecosystems are constantly threatened by climate change and the indiscriminate use of pesticides. The metabarcode analysis of eDNA (environmental DNA) method is highly effective at accumulating information on many organisms that inhabit paddy field ecosystems. It can indirectly identify the existence of taxa that are no longer found in the target ecosystem due to behavioral characteristics, such as those exhibited by amphibians. In the metabarcoding results of this study, genes of five species of frogs were found, but it was impossible to confirm all of the frogs' taxa, morphological pictures, and croak sounds. On the other hand, some frog taxa were only found in the metabarcoding analysis. The eDNA of the frogs found only in the metabarcoding analysis is estimated to have been introduced from nearby areas inhabited by frogs rather than the target region. Due to the powerful analytical resolution of eDNA metabarcoding, this eDNA-based paddy field search is expected to help investigate the biodiversity in agricultural ecosystems.

Keywords: environmental DNA; metabarcoding; paddy field; amphibia; biodiversity

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1. Introduction

A paddy field ecosystem is an artificial wetland created inland when water is supplied during a specific season (from spring to summer), and it is a unique form of wetland ecosystem connected to rivers and reservoirs where various aquatic species spawn and thrive [1,2]. The paddy field ecosystem, accounting for approximately 18% of all wetlands on Earth, is a crucial component of a comprehensive plan that utilizes wetlands to address environmental pollution and preserve biodiversity worldwide. Since the Ramsar Convention adopted "Enhancing biodiversity in rice paddies as a wetland system", there has been a growing interest in promoting biodiversity using paddy fields [3,4]. Furthermore, biodiversity has significant implications in terms of biological pest control and agricultural productivity [5–7]. The biodiversity of pests demonstrates a close relationship with agricultural production. The diversity of organisms involved in pest control, such as spiders and frogs, reflects a close connection to the sustainability of agricultural systems.

Most of the biological surveys conducted on paddy field ecosystems aim to understand the status of agricultural pests. In the early days, research surveys were mainly conducted to determine the incidence of mosquitoes in paddy fields, and a full-fledged research survey on aquatic organisms in paddy fields was conducted in 1994 [8]. Moreover, since the Ramsar Convention in 2008, NGOs have also formed networks and conducted biological surveys of paddy fields [8–10]. Even today, surveys are being conducted on the actual condition of paddy field ecosystems to investigate the emergence of amphibians, reptiles, spiders, and aquatic insects living there and to identify the date and time of the appearance of different types of biota due to climate change and regional changes [11,12].

Amphibians serve as predators in agricultural environments by feeding on agricultural pests, and this ecological role has been utilized in biological pest control [13,14]. In particular, orthopteran insects such as grasshoppers and locusts cause direct damage to crops, and frogs that inhabit paddy fields help prevent pest damage by preying on these species [15]. Therefore, the species diversity of amphibians in paddy environments is highly valuable as a fundamental indicator of the health of paddy ecosystems and pest control in agricultural environments. Additionally, frogs are sensitive to climate change and pesticide use, making them important indicator species for monitoring climate change and excessive pesticide use [16–18].

In general, state surveys of organisms in these paddy field ecosystems utilize a traditional method that involves directly collecting and observing individuals. Amphibians, like frogs, are identified by investigators through the direct filming of individuals on the spot or by listening to their croak sounds [19]. However, these traditional collection methods are highly labor-intensive and they heavily rely on the timing of the investigation as well as on the investigator's competence. Moreover, conducting a survey by searching for individuals in a wide range can be time-consuming, and if the population is small, identifying individuals in paddy field ecosystems becomes highly challenging.

The organism screening method using environmental genes was proposed as a solution to the problems and difficulties associated with traditional investigation methods [12–22]. Environmental DNA (eDNA) refers to the genetic material that exists in large quantities in various environments, such as water, air, and soil, and this genetic material includes traces of organisms living in the environment [23–25]. Thus, it can be used as a clue to determine the existence of organisms in the environment without the need for collecting them directly. Currently, organism screening using eDNA is most actively used in fish taxa, and it is also actively utilized to search for endangered, foreign, and harmful species [25–27]. Biodiversity has been primarily identified in river and lake environments using eDNA, with only a few studies exploring biodiversity using eDNA in paddy field ecosystems [28–31]. Amphibians, such as frogs, in particular, live, spawn, and reproduce around paddy fields or nearby ecosystems, which means that their genetic traces may exist within paddy field ecosystems. Frogs, as amphibians, require water for breeding and refuge from predators. The paddy field ecosystem, classified as a freshwater habitat, serves not only as a breeding ground for frogs but also plays a crucial role as a hiding place. In the paddy field ecosystem, frogs occupy a vital ecological niche as predators of pests such as rice planthoppers.

Therefore, this study utilizes eDNA to identify and verify the frog community residing in the aquatic ecosystem of a paddy and stream watershed connected to a paddy field in order to assess the applicability of eDNA for screening the biodiversity of the aquatic ecosystem of an agricultural environment.

2. Materials and Methods

2.1. eDNA Sampling and Extraction

The surveyed area of this study is centered around small-scale paddy fields (site U; Upper paddy: 37°14′12.44″ N 127°13′6.51″ E, site L; Lower paddy: 36°13′57.5″ N 127°13′19.88″ E), surrounded by forested areas (Figure 1). With two small streams flowing in proximity to the paddy fields, the surrounding environment facilitates the access of organisms to the fields during their breeding and mating seasons. Furthermore, the low residential density in the vicinity of the paddy fields results in relatively minimal human-induced disturbance.

We conducted eDNA sampling once during a rain-free period lasting one week in May. We aimed to prevent confusion caused by external sources of eDNA entering during the rainy season and to minimize the loss of eDNA due to increased water flow. Considering the activity characteristics of amphibians, eDNA was collected from the water at sunset and immediately filtered and collected on-site using Dual eDNA Filter™ (Sphylum, Groningen, The Netherlands; 0.45 μm pore size) and a syringe (max volume: 60 mL). A single filter was used for each area of the paddy, and eDNA was collected from at least three points,

including the central and outskirt areas of the paddy. The sampling was conducted by a collector, who wore boots and entered the site directly. While monitoring the real-time location, they initiated filtering from the outer edge of the field and moved inward at 1 m intervals, filtering 100 mL at each single point, and obtained the concentration of eDNA present over 300 mL of water from a single paddy field. One filter and syringe were used to concentrate the eDNA in a single field, and the syringe and filter were replaced when moving to different fields. To prevent contamination during the eDNA collection process, Nitrile gloves were consistently worn, and sterilized syringes were used for each paddy field to avoid cross-contamination. Furthermore, to prevent contamination through field collection attire (chest waders), a two-step cleaning process was employed. The clothing was initially washed with tap water and then subjected to a secondary wash with a 3% dilution of sodium hypochlorite to minimize contamination between sampling points. eDNA was collected from a nearby stream using the same sampling process that was used for the paddy. Immediately after collection, the outlet of the filter was sealed with a Luer lock and transported to the laboratory while being maintained at a low temperature (4 °C). Extraction of eDNA was performed within 24 h.

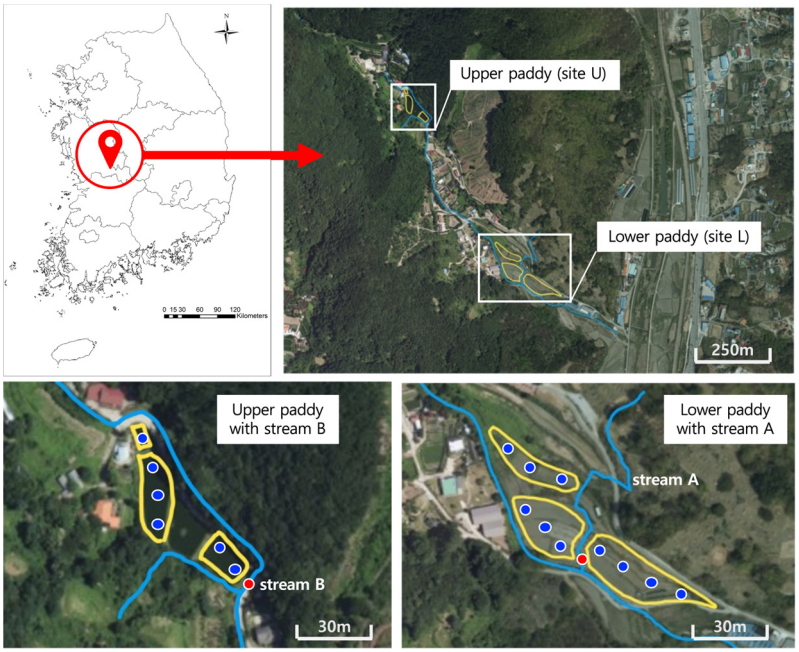


Figure 1. Survey region of this study. The part was separated into the following two areas: upper and lower paddies. On the map, the blue circles represent points where eDNA sampling was conducted in the fields, while the red circles indicate points where eDNA was collected from the river. The yellow line represents edge of each paddy and the blue line indicate streams.

The dual eDNA filter was transported to the laboratory using the Manual Environmental DNA Isolation Kit (#SYL002, Sylphium molecular ecology Ltd., Groningen, The Netherlands) following the "Procedure of DNA isolation from water (eDNA dual filter capsule)" to extract the eDNA, and the extracted eDNA was then stored in a deep freezer (−80 °C) before analysis.

2.2. PCR Amplification

To identify the genes of amphibians present in the eDNA of the aquatic ecosystem in paddy fields, a primary PCR of the mitochondrial 16S rDNA (mt16S rDNA) gene was conducted using the primer (Amph16S_1070_F and Amph16S_1340_R) presented by Sakata et al. [32]. The DNA polymerase used for this purpose was the 10 µL of 2× GainBlue™ Hot Start Max Master Mix PCR Master Mix (Gainbio, Daejeon, Republic of Korea). Forward and reverse primers were added at a concentration of 10 pmol/µL (1 µL was the volume of

each primer), and 5 µL of nuclease-free water was added. Finally, 3 µL of template eDNA was added, and PCR was performed using the PCR Thermal Cycler (LongGen, Taipei, Taiwan). Sterilized distilled water was added alongside the sampled eDNA from the paddy field, serving as a substitute for template DNA. This was employed as the negative control group in the study. Thermal cycling consisted of initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 20 s followed by a final extension at 72 °C for 5 min. The product of primary PCR amplification was analyzed using the 2% agarose gel of E-gel^{TM} Power Snap Electrophoresis system (Thermo Fisher Scientific; Waltham, MA, USA) to perform electrophoresis, and the amplification product was then identified using an E-gel^{TM} Power Snap Camera (Thermo Fisher Scientific, USA).

2.3. eDNA Metabarcoding Index PCR and Bioinformatics Analysis

For metabarcoding analysis, electrophoresis was performed on the primary PCR amplification product using a 2% size selection gel of the E-gel^{TM} Power Snap Electrophoresis system (Thermo Fisher Scientific, USA), followed by gel extraction of the target amplification product. The primary PCR amplification product was separated through gel extraction, and then a barcode index (Illumina Nextrea kit V2; San Diego, CA, USA) was attached through a secondary PCR process. The second PCR consisted of initial denaturation at 95 °C for 3 min followed by 8 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 20 s, and elongation at 72 °C for 20 s, followed by a final extension at 72 °C for 5 min. Samples with the barcode index were analyzed using metabarcoding with approximately 30,000 reads per each sample based on the Illumina MiSeq System by a company specializing in analysis (Ubix bioinnovation Co., Ltd., Waterloo, ON, Canada). Demultiplexed sequences were processed using Cutadapt (primer removal) DADA2 v.1.22 (sample inference) [33,34]. The Cutadapt and DADA2 analyses were performed using the following parameters: maxN = 0, maxEE = c(5,5), truncQ = 2, minLen = 50, truncLen = c(240—(forward primer length) and 225—(reverse primer length)). Reads were truncated at decreasing quality scores and assembled. After quality filtering (chimeras and organelles) and taxonomic assignment, an abundance table was constructed. Species identification based on metabarcoding results was conducted using a 99% identity threshold. This abundance table records the number of sequence reads for each amplified sequence variant ("species"; row) for each sample (column). Taxonomy was assigned to representative sequences using IdTaxa from the DECIPHER package [35] trained against a reference database (16S/18S: SILVA release 138 at 99%, ITS: UNITEdb ver. 8 dynamic) [36–38]. The data analyzed from each paddy field were divided and analyzed based on the upper and lower paddies.

2.4. Field Survey of Frogs

Individuals found based on eDNA were verified by directly observing them in the area where eDNA was collected or by identifying their cry. The survey was conducted in accordance with the amphibian survey method provided in the "Standards and Manuals for Ecological Environment Survey and Evaluation in the Agricultural Sector" to identify amphibians living in the paddy field environment [19]. In this study, the traditional frog occurrence survey was conducted both during the day and at night. The daytime survey was conducted for 2 h starting at 10 a.m., and the nighttime survey was conducted for 2 h starting at 9 p.m. Each survey was conducted for 1 h in the upper paddy and 1 h in the lower paddy, for a total of 2 h. The surveys were conducted during the day and at night on the same day as the eDNA sampling. Frogs were observed while walking along the ridges and paths of the paddies, and both the species and the number of individuals were recorded. Frog presence was assessed based on both visual observations and auditory cues. During the daytime, adult frogs were visually examined in paddy fields, along the ridges between paddies, and in waterways within the investigated area, and species were identified by taking pictures of the individuals [39].

3. Results

3.1. Amphibian Diversity Based on eDNA Metabarcoding

As a result of analyzing the genes of the frogs included in Anura's mitochondrial 16S rDNA (mt 16S rDNA) in the eDNA collected from the paddy environment, we detected the DNA of five species of frogs, including *Pelophylax nigromaculatus*, *Hyla japonica*, *Glandirana rugosa*, *Rana uenoi*, and *Rana huanrenensis* (Figure 2, Table 1). The proportion of frog gene contigs detected at each site was calculated based on the contig numbers of frog genes analyzed through metabarcoding.

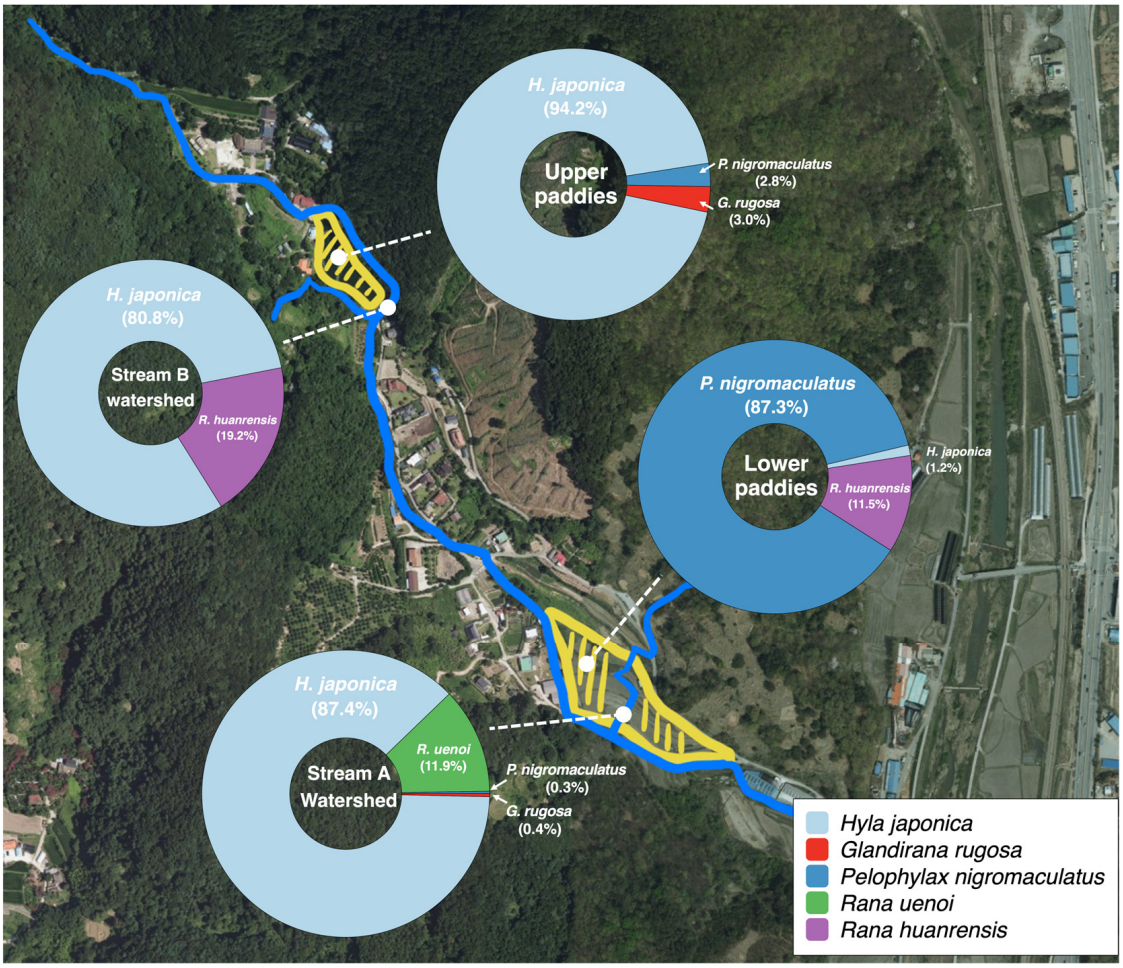


Figure 2. Metabarcoding community analysis of the mitochondrial 16S rDNA sequence in paddies and streams. The results of the frog eDNA analysis conducted at each point in the paddies were categorized into upper and lower sections of the paddy.

Table 1. Contig numbers of frog genes detected by metabarcoding in the survey area.

Contig Number of Species	Lower Paddies	Stream A	Upper Paddies	Stream B
Hyla japonica	13	844	42,700	4622
Pelophylax nigromaculatus	931	0	1253	13
Glandirana rugosa	0	0	1380	20
Rana uenoi	0	0	0	631
Rana huanrenensis	123	200	0	0
Bufo gargarizans	0	0	0	0
Total contig number	1067	1044	45,333	5286

In the upper area (the upper paddies and stream B), we detected a total of four frog species in the paddies and stream. In the upper paddies (site U), three species with frog genes were found, while in the streams, two species with frog genes were found. The *H. japonica* DNA accounted for the largest proportion of the frog mt 16S rDNA in the upper area, with 94.2% and 80.8% in the paddies and stream, respectively. Relatively small proportions of *P. nigromaculatus* and *G. rugosa* were found at 2.8% and 3.0%, respectively, and only in the paddies. In stream B, *P. nigromaculatus* and *G. rugosa* were not found, and *R. huanrenensis* DNA accounted for 19.2% of the frog mt 16S rDNA. The *H. japonica* DNA had a different pattern in stream B than in the paddies.

In the lower area (the lower paddies and stream A), the genes of *R. uenoi* were found only in the stream, and they accounted for a higher proportion than the genes of *G. rugosa* and *P. nigromaculatus*. On the other hand, at the lower paddies (site L), we detected a total of five frog species in the paddies and stream; in the paddies, the genes of three frog species were found, while in the stream, the genes of four frog species were found, exhibiting differences from the frog clusters in the upper area. In contrast, at site U, the DNA of *P. nigromaculatus* accounted for the largest proportion with 87.3%, while *H. japonica* accounted for the lowest proportion with 1.2% in the paddies. In addition, the DNA of *R. huanrenensis*, which was not found at site U, accounted for 11.5% at site L. The DNA of *G. rugosa*, which was detected in site U, was not detected in site L. In the waterways near site L, similar to the upper area, the proportion of the DNA of *H. japonica* was the highest with 87.4%, and the proportion of the DNA of *R. uenoi* accounted for 11.9%. Additionally, in stream A, the genetic proportions of *P. nigromaculatus* and *G. rugosa* were both below 1%, representing very low percentages, which showed a stark contrast to the proportions found at the adjacent site, site L.

3.2. Verification of eDNA Detection by Field Survey

The results of the eDNA investigation were verified by comparing the species of frogs found using eDNA with the species identified through direct observations and croak sounds (Supplementary Datas S1 and S2). Adult frogs and tadpoles were found in two areas of the paddy fields and streams through direct observations of individuals and by identifying frog species living at the investigated site through their calls, but the list of species directly observed did not completely match with the list of species in the eDNA-based frog community (Figure 3). Furthermore, the population ratios of the observed frog species in the surveyed area did not align with the gene contig ratios of the frog species identified through the metabarcoding analysis.

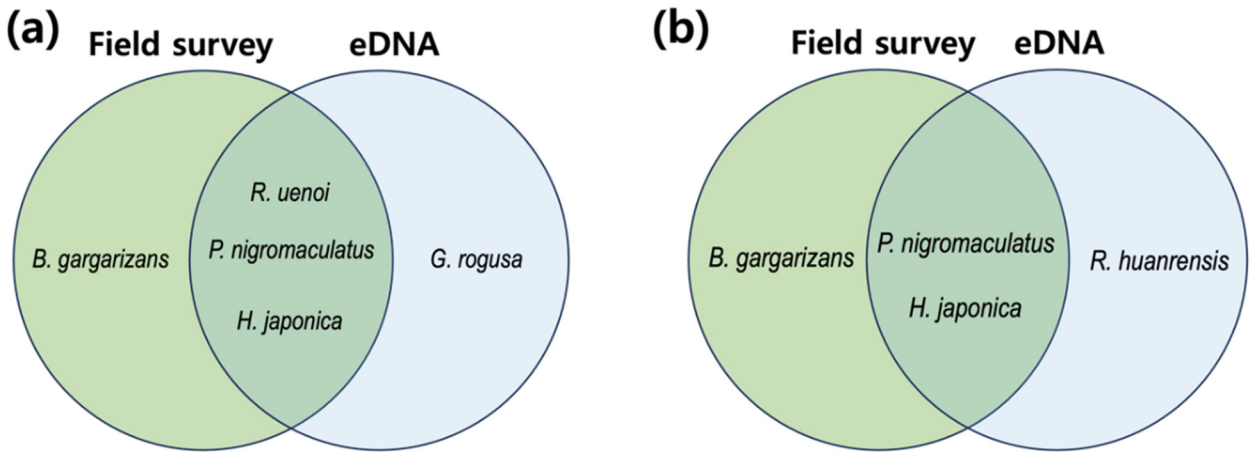


Figure 3. Comparison of eDNA and traditional survey for paddy field frog detection. We verified the upper paddy region and lower paddy region. Each circle indicates results of detection by eDNA and conventional field survey method. (**a**) Means validation of upper paddy region and (**b**) means validation of lower paddy region.

At site U, three adults of *H. japonica* and four adult individuals of *P. nigromaculatus* were spotted, and one adult individual of *R. uenoi* was found in a puddle upstream of the upper paddies. *G. rugosa* were not found inside the upper paddies, and their croak sounds were not identified either. Thirty adult individuals of *H. japonica* were found at site L, but only eight individuals of *P. nigromaculatus* were found—fewer than the number of adult *H. japonica* individuals. *R. huanrenensis*, whose DNA was found in the lower paddies, was not found in the paddies or waterways, and their croak sounds were not heard. At the investigation site, *H. japonica* were primarily discovered on the ridges surrounding the paddies and around the paddies, with adult *H. japonica* individuals primarily being located within the paddies. *R. uenoi* were found around the puddle upstream rather than around the paddies. Adult frogs were not found in the waterways at the investigation site, and twelve larvae (tadpoles) of *R. uenoi* were discovered. In the process of searching for adult frogs, one toad was directly identified in the area between site U and site L, and adult toads or toad larvae were not found in the paddies or waterways.

4. Discussion

4.1. eDNA Soup in Agricultural Aquatic Ecosystems

Agricultural water is supplied through irrigation systems from agricultural reservoirs or nearby streams. Therefore, in the agricultural water of paddy fields, the DNA of organisms living in the upper stream of the reservoirs and that of organisms living in paddy fields coexist [40–44]. In this study, neither *G. rogusa* nor *R. huanrenensis* were identified through direct observations or croak sounds, and a very low proportion of their DNA was identified. Hence, identifying DNA from *G. rogusa* and *R. huanrenensis*, which were not found in the paddy fields, suggests that individuals from these species likely inhabit areas outside of the paddy fields or in the upper regions [45–48]. Furthermore, the two paddies in the study area do not contain water year-round. The lower paddies, in particular, were newly created in the year the study was conducted and had been used as a dry field without water in the previous year. This makes the area unsuitable as a habitat for frogs, which is likely why no *G. rugosa* and *P. huanrenensis* individuals were detected in the survey area. In other words, the DNA of frogs of *G. rogusa* and *R. huanrenensis* residing in the upper watershed areas may have been introduced to the paddy fields through the irrigation of streams. In addition, *R. uenoi* and *G. rogusa* were found only in the upper sites, while the DNA of *R. huanrenensis* was found exclusively in the lower paddies and the stream. This suggests that the DNA of organisms, such as frogs and fish, that roam within their boundaries can also be found in areas near their habitat, and even the DNA collected from paddy fields in the same area may vary depending on the specific location where the eDNA was collected [49–51]. Therefore, the DNA of *R. uenoi* and *G. rogusa* found only in the upper sites suggests that these two frogs live exclusively in the upper part of the stream. Also, *R. huanrenensis* are believed to inhabit the stream of the valley that is irrigated to the lower paddy, with their DNA flowing into the stream near the lower sites.

4.2. Differences in eDNA Release Based on Ecological Characteristics

The number of amphibians is not proportional to the number of contigs in eDNA, and the number of contigs in eDNA differs depending on the ecological characteristics of each taxon [52–54]. In this study, although there were more than twice as many *H. japonica* in the lower paddies, a higher amount of DNA from *P. nigromaculatus* was collected in the eDNA of the water samples. After undergoing metamorphosis from tadpoles to adults, *H. japonica* are primarily active in areas with leaves, grass, and shrubs, with their presence in the paddy fields limited to the breeding season [55–58]. In contrast, the habitat of *P. nigromaculatus* is closely related to the freshwater environment. *P. nigromaculatus* have a protective color similar to that of paddy water, and they hide in aquatic plants, such as duckweeds, which are present in the paddy fields [59]. Thus, although the population of *H. japonica* was higher around the paddy fields, it is believed that the DNA of *P. nigromaculatus* dwelling underwater accounted for a larger proportion than that of *H. japonica*. However, in the

upper paddies, *H. japonica* accounted for a larger proportion of genetic contigs, despite the similar population sizes of *H. japonica* and *P. nigromaculatus*. This is believed to be because the DNA of *H. japonica*, which constitutes a significant proportion of the stream, flowed into the paddy fields, thereby affecting the proportion of *H. japonica* DNA in the fields. In addition, *R. uenoi* mainly inhabit the mountain slopes, streams, and arable land in the valley, and they spawn in still water rather than in flowing water [59,60]. The discrepancy between the population ratios and gene contig ratios raises concerns about the practicality of using a metabarcoding analysis for the quantitative assessment of frog clusters. Consequently, utilizing eDNA from agricultural environments for accurate quantitative analyses of frog populations may prove challenging, while qualitative analyses remain feasible.

In this research, a few individuals were also found near water pools located upstream of the fields. However, given that *R. uenoi* constitute the second largest proportion of DNA found in the upstream water, it is estimated that there will be a greater abundance of *R. uenoi* in the upper valley at the survey site. Moreover, although their DNA was not found in the eDNA of the survey site, *Bufo gargarizans* (toads) were directly observed and identified at the site, which mainly crawl on land as adults and breed in groups only during the spawning season [61]. *B. gargarizans* spawn in still water between March and April, but during the research period, there was no water in the paddy fields during this season at the survey site. Therefore, *B. gargarizans* did not spawn in paddy fields at the survey site of this study, and it is estimated that they spawned in puddles located outside the paddy fields.

5. Conclusions

The aquatic ecosystems in paddy fields are used as spawning grounds and habitats for organisms, such as frogs. The use of eDNA can help researchers search for traces of organisms at the DNA level, allowing for the detection of organisms in paddy fields without direct observations of individuals. In this study, it was possible to determine the presence of creatures living adjacent to paddy fields by detecting the DNA of *R. huanrenensis* and *G. rogusa*, which were not observed directly. In addition, research has led to the possibility that more individuals of *R. uenoi* could live upstream of site U. This biological exploration of paddy fields using eDNA provides the advantage of a high analytical resolution, making it a valuable method for studying the biodiversity of the agricultural ecosystem. However, due to the nature of the paddy field environment, various organisms pass through and DNA from humans (*Homo sapiens*) is introduced when planting and managing rice; thus, the DNA introduced from external sources can impact the analysis of biological communities at the survey site. In addition, similar to the case of toads, it can be very difficult to identify the DNA of organisms that live mostly on land, except during the larval period, in eDNA research. Therefore, eDNA-based surveys on biological communities and on-site censuses should be treated as complementary research methods that can interact with each other, rather than as separate research methods. In other words, to supplement the search results at the DNA level through eDNA, it is necessary to extract the list of species observed at the survey site and proceed with direct observations and investigations. Hence, the future utilization of both eDNA and traditional direct observation methods is expected to yield crucial data for confirming biodiversity in paddy field ecosystems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w16213063/s1, Supplementary Data S1: Field survey results of the study area. A circle indicates the frog individuals directly observed in the area. A speaker mark means heard croaking sound of frogs in the area. In the study area, we could not hear the croaking sound of *R. huanrenensis* and only found an adult individual. Supplementary Data S2: The photographs of frogs that were taken in the study area. (a–c) *R. huanrenensis* adult and tadpole were found near the pond and stream. (d–e) *H. japonica* adult individuals near the paddies. (f–i) *P. nigromaculatus* near the paddies and in the paddies. (j) *B. gargarizans* adult individual that was found outside the paddies.

Author Contributions: K.K. confirms responsibility for the following: conceptualization and methodology, formal analysis, data curation, supervision, project administration, and manuscript preparation. Y.J. and S.K. were responsible for contributing to the formal analysis and validation with the field investigation. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data used and analyzed during this study are available from the corresponding author on reasonable request.

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