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Topographic Wetness Heterogeneity within Paddy Landscapes Facilitates the Coexistence of Closely Related Species

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Abstract

Paddies have long served as alternative habitats to natural wetlands and support a wide variety of organisms. However, urbanization and agricultural abandonment have caused substantial losses of paddy fields, resulting in biodiversity declines. As paddy landscapes become increasingly restricted and homogenized, closely related species may be forced to coexist within the same habitats, potentially increasing interspecific interactions. Understanding the environmental conditions that facilitate species persistence and coexistence is therefore important for biodiversity conservation in paddy-field landscapes. In this study, we used environmental DNA (eDNA) analysis to investigate landscape conditions promoting the coexistence of two closely related frog species, the Nagoya daruma pond frog (*Pelophylax porosus brevipodus*) and the Black-spotted pond frog (*P. nigromaculatus*). Water samples were collected from 87 paddy fields in three river basins in Fukui Prefecture, Japan, and species occurrence was determined using species-specific real-time PCR assays. We then applied a joint species distribution model (JSDM) to identify environmental factors influencing species distributions and to evaluate potential interspecific interactions through residual correlations. The results showed that the eDNA detection probability of *P. p. brevipodus* was mainly positively associated with the topographic wetness index (TWI), whereas that of *P. nigromaculatus* was negatively associated with TWI, indicating contrasting responses to wet environmental conditions. Residual correlation between the two species was close to zero, indicating little evidence of strong interspecific interactions. Our study highlights the importance of wetness heterogeneity for the coexistence of two closely related species and the maintenance of biodiversity in paddy-field landscapes.

Introduction

Paddies have long served as alternative habitats to natural wetlands worldwide and have supported a wide variety of organisms (Natuhara 2013; Propper et al. 2023). However, biodiversity in paddy field ecosystems has declined due to the spread of modern intensified agricultural practices, including land consolidation with associated drainage improvement and increased use of pesticides and chemical fertilizers (Fujioka and Lane 1997; Katayama et al. 2015). Therefore, the conservation of biodiversity-rich paddy field environments has become an urgent priority, and numerous studies have investigated the effectiveness of biodiversity-friendly farming practices (Katayama et al. 2020; Propper et al. 2023).

The global crisis facing paddy field ecosystems is driven not only by the degradation of paddy field environments but also by the loss of paddy fields themselves (Koshida and Katayama 2018). In Japan, paddy fields located on mountainous slopes are particularly prone to abandonment because agricultural mechanization is difficult (Uematsu et al. 2010; Qiu et al. 2014), whereas paddy fields in lowland areas have been rapidly lost to urban development since the 20th century (Tsuji et al. 2011; Suozhu et al. 2020). Such reductions in paddy field area, together with the loss of environmental heterogeneity, have led to declines in biodiversity within paddy field ecosystems (Natuhara 2013; Koshida and Katayama 2018). Furthermore, as paddies have continued to disappear, closely related species that historically partitioned spatial niches may increasingly be forced into sympatry within the same paddy fields,

potentially intensifying negative interspecific interactions such as resource competition and reproductive interference (Nakanishi et al. 2020; Ye et al. 2021; Sawada et al. 2025). Given the ongoing decline in paddy field area across Japan, understanding landscape-level environmental heterogeneity that promotes the persistence and coexistence of paddy-associated organisms is also important, as such knowledge can contribute to the efficient conservation and restoration of paddy field landscapes (Nakanishi et al., 2020; Ogura et al., 2025; Watanabe et al., 2019; Yamazaki and Nishio, 2025; Ogura et al., under review).

To address this issue, we targeted two closely related, threatened frog species that are strongly associated with paddy fields and examined the landscape factors facilitating their coexistence within restricted paddy field landscapes. Specifically, we focused on two congeneric species that are highly dependent on paddy fields as breeding, foraging and overwintering habitat: *Pelophylax porosus brevipodus* (Nagoya daruma pond frog) and *P. nigromaculatus* (Black-spotted pond frog). The geographical distributions of these species partially overlap, and mutual reproductive interference, including heterospecific amplexus and subsequent hybridization, has been often reported in areas where they coexist (Shimoyama 1999; Shigeta et al. 2025). A broad-scale study conducted around Lake Biwa, showed that *P. p. brevipodus* primarily inhabits lowland areas, whereas *P. nigromaculatus* occurs in hilly areas or in lowlands where *P. p. brevipodus* is absent (Nakanishi et al. 2020). However, given the ongoing nationwide decline in paddy field area throughout Japan, their coexistence patterns and underlying mechanisms should be clarified not only in lowland landscapes but also in mountainous areas, such as valley-bottom plains, because such areas now represent one of the few remaining habitats for both species.

In this study, we investigated the distributions of two *Pelophylax* species in three river basins in Wakasa Town, Fukui Prefecture, and examined landscape-scale, habitat partitioning patterns use in areas where the two species coexist. Conventional surveys of frog distributions have relied on capture, visual observations, and acoustic methods, but each of these methods has several limitations. The former two methods are labor-intensive, making large-scale surveys difficult (Ogura et al. 2025). Acoustic surveys are often unable to accurately determine the locations and abundance of calling individuals (Niwa and Uno 2023), and detection rates may decline after the breeding season in *P. nigromaculatus*, which has a short breeding period (Nakanishi et al. 2020). To overcome these limitations, we used environmental DNA (eDNA) analysis. Due to its high sensitivity, this method is well suited for efficient and noninvasive surveys of amphibians with cryptic lifestyles (Sun et al. 2024). Furthermore, unlike conventional survey methods that primarily target adults, such as capture and call surveys, it can also detect larvae, making it less susceptible to seasonal constraints. Using this approach, we conducted a comprehensive survey of the distributions of both species throughout the study area.

Material and Methods

Study species

The target species were two closely related congeners, *P. p. brevipodus* and *P. nigromaculatus*, both of which use paddies as important habitats for breeding, foraging and overwintering. *Pelophylax p. brevipodus* mainly breeds in paddies from late April to mid-July and inhabits paddies even during the non-breeding period (Matsui 2018). In contrast, *P. nigromaculatus* breeds from April to June and some individuals leave paddies during the non-breeding period (Matsui 2018).

Field survey

Water sampling surveys were conducted over five days in June 2025 in 87 paddies across the Hasu, Toba, and Kitagawa River basins in Wakasa Town, Fukui Prefecture, Japan (35.47–35.54°N, 135.87–135.91°E; Fig. 1), after obtaining prior consent from the paddy owners. At each site, we collected water samples at the outlet of paddies. In two paddies where water bodies were too small to allow outlet sampling, samples were instead collected from adjacent irrigation canals; however, these were used only for mapping species distributions and were excluded from subsequent analyses.

At each site, 500 mL of water was collected in a plastic bottle, and 0.5 mL of 10% benzalkonium chloride solution was added to preserve eDNA (Yamanaka et al. 2017). As field blanks, 1 L of purified water was prepared for each sampling day and processed in the same manner as environmental samples. On the second sampling day, field blanks were not collected; instead, a filtration blank consisting of purified water processed through the filtration apparatus was used as a negative control.

Water samples were filtered using two glass fiber filters (250 mL per filter). Environmental DNA retained on the filters was extracted using the DNeasy Blood & Tissue Kit (QIAGEN) following the protocol outlined in the eDNA Society's experimental manual (Minamoto et al. 2021).

Real-time PCR analysis

To simultaneously detect the two target species, multiplex real-time PCR was performed using a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, USA). The real-time PCR assay for *P. p. brevipodus* was newly developed in this study (see Text S1 for details of primers and probe design), whereas for *P. nigromaculatus*, we adopted a previously designed assay developed by the authors (Ogura et al., under review) (Table 1).

Table 1
Sequences of primers and probes used in this study

Target species	Primer / probe name	Sequence (5'>3')	Amplicon length
Pelophylax porosus brevipodus	Ppb-Dloop-F	ATGGACCTGCATAAAGTATAATAACCG	179 bp
	Pp-Dloop-R	ATTAAGGTCCATTGGAAAATTAAGATCATC	
	Ppb-Dloop-P	HEX-CCCGTACAATCACCACGACGCGTAC-BHQ	
Pelophylax nigromaculatus	Pnigro-Cytb-F	CTCTCAATCATAGTCCTCTTTCTGA	119 bp
	Pnigro-Cytb-R	AGAATGGCTGTATTGGCAATTAGTG	
	Pnigro-Cytb-P	FAM-AACTGCGCTCCCTGATGTTTCGCC-BHQ	

Each reaction mixture (20 μL total volume) contained 2 μL of template DNA, 1× TaqMan Environmental Master Mix 2.0, 0.1 μL of AmpErase Uracil N-glycosylase, 900 nM of each primer, 125 nM of TaqMan probe, and ultrapure water. As positive controls, tissue-derived DNA from each species adjusted to 5 pg/μL was used. Non-template controls (NTC) consisted of reactions in which template DNA was replaced with ultrapure water.

Each sample was analyzed in triplicate. Thermal cycling conditions consisted of 50°C for 2 min and 95°C for 10 min, followed by 55 cycles of 95°C for 15 s and 60°C for 1 min. Field blanks and filtration blanks were processed using identical procedures to those applied to environmental samples.

Environmental variables

To analyze differences in landscape-scale habitat use between the two species, environmental variables related to land use and topography were compiled. Buffers with radii of 25, 50, 75, and 100 m were created around the perimeter of each paddy field. Within each buffer, the areas of paddies, green spaces (e.g., crop fields, grasslands, gardens and forests), and artificial structures (e.g., buildings and roads) were calculated. Land cover was interpreted using seamless aerial photographs provided by the Geospatial Information Authority of Japan (https://www.gsi.go.jp/ENGLISH/, accessed June 17) and Google Maps (https://cyberjapandata.gsi.go.jp; https://www.google.com/maps/, accessed June 17).

The Topographic Wetness Index (TWI), representing topographic variation in water accumulation potential, was obtained from datasets provided by the Biodiversity Center of Japan (https://www.biodic.go.jp/, accessed June17) and averaged within each buffer. As buffer-independent variables, the mean slope angle of each paddy field location was extracted from the “Elevation and Slope

5th Mesh Data" provided by the National Land Numerical Information download service. Additionally, the distance from each sampling point to the nearest forest edge was included.

Collinearity among all explanatory variables was checked and confirmed to be low (variance inflation factor, VIF < 3). All spatial analyses were conducted using QGIS version 3.34 (QGIS Development Team, 2019).

Statistical analysis

To evaluate differences in habitat use between the two congeneric species while accounting for interspecific interactions, we constructed generalized linear latent variable models (GLLVMs). GLLVMs incorporate latent variables in addition to fixed effects to account for residual covariance among species that cannot be explained by environmental variables, including unmeasured environmental factors and interspecific interactions (Niku et al. 2019).

The response variable was the number of positive real-time PCR replicates (0–3) for each species, modeled using a binomial distribution. Seven environmental variables were included as explanatory variables, and separate models were constructed for each buffer scale (25, 50, 75, and 100 m). All explanatory variables were standardized using Z-scores to allow comparison of effect sizes. Because the analysis included only two species, the number of latent variables was fixed at one. This latent variable was used to estimate residual species correlations after controlling for environmental variables, spatial structure, and random effects. Residual correlation represents the degree of association between species that remains unexplained by the measured variables and may reflect unmeasured ecological processes, such as unobserved environmental gradients and interspecific interactions. As a random effect, river basin was included. Because the Hasu River basin is geographically separated from the others by mountainous terrain, whereas the Toba and Kitagawa River basins are not clearly separated, the latter two were combined into a single basin category. To account for spatial autocorrelation, geographic coordinates of sampling locations were incorporated into the models. Buffer size selection was performed using Akaike's Information Criterion (AIC), and the model with the lowest AIC was selected as the best buffer size.

All statistical analyses were conducted using R (R Core Team, 2024). GLLVMs were fitted using the gllvm package (Niku et al. 2019), and visualization was performed using ggplot2 (Wickham 2016).

Results

Distribution patterns revealed by eDNA analysis

Positive eDNA signals for *P. p. brevipodus* and *P. nigromaculatus* were detected in 30 and 56 samples, respectively, out of 87 samples (Fig. 2). DNA of *P. p. brevipodus* was primarily detected around Lake Mikata in the Hasu River basin, with only a single detection in the Toba River basin outside this area. In

contrast, DNA of *P. nigromaculatus* was sparsely detected throughout the study region. No amplification was observed in any of the field blanks, filtration blanks, or NTC.

GLLVM analysis of environmental effects and interspecific correlation

Buffer size selection based on AIC indicated that the model using environmental variables calculated within a 75 m buffer provided the best fit (Fig. S1). The GLLVM result showed that the number of positive real-time PCR replicates for *P. p. brevipodus* was significantly positively associated with the TWI within a 75 m buffer (β = 0.75, $p < 0.01$) and was significantly negatively associated with distance to the forest edge (β = -0.37, $p < 0.05$) (Fig. 3a; Table 2). For *P. nigromaculatus*, the number of positive real-time PCR replicates was significantly negatively associated with the TWI (β = −1.07, $p < 0.01$) (Fig. 3a; Table 2). The residual correlation between the two species, after accounting for environmental variables, spatial autocorrelation, and river basin effects, was low and positive ($r = 0.076$).

Table 2
Effects of environmental variables on two frog species (*Pelophylax porosus brevipodus* and *Pelophylax nigromaculatus*) estimated using a generalized linear latent variable model (GLLVM) with a 75 m buffer.

	P. p. brevipodus			***P. nigromaculatus***		
	Estimate	**Pr(>|z|)**		**Estimate**	**Pr(>|z|)**	
TWI	0.75	< 0.001	***	-1.07	< 0.001	***
Area of paddy fields	0.00	0.98		-0.01	0.96	
Area of green space	-0.10	0.47		-0.21	0.34	
Area of artificial structures	0.07	0.57		0.37	0.09	
Slope	-0.19	0.23		0.10	0.62	
Disrance to the forest edge	-0.37	< 0.05	*	-0.30	0.24	

For each explanatory variable, the estimated coefficient (Estimate) and p-value (Pr (>|z|)) are shown. Asterisks indicate levels of statistical significance: *** p < 0.001, * p < 0.05, and. p < 0.1.

Discussion

Wetness gradient plays an important role in coexistence

The detection probability of eDNA for *P. p. brevipodus* showed a positive relationship with TWI, whereas *P. nigromaculatus* exhibited a negative relationship, indicating contrasting responses between the two species (Fig. 3a). The relatively greater dependence of *P. p. brevipodus* on paddy fields, compared with

the lower dependence of *P. nigromaculatus*, may reflect character displacement that facilitates the sympatric coexistence of the two species.

Pelophylax p. brevipodus is highly dependent on wet paddy field conditions (Matsui 2018), and its population has declined nationwide due to drying during the non-irrigation period following land consolidation (Naito 2012). Although land consolidation has also been widely implemented in the study area, rainfall during the non-irrigation period may mitigate the drying of paddies. In particular, areas with high TWI tend to have poor drainage, suggesting that suitable moist habitats for *P. p. brevipodus* may persist even outside the irrigation period. In addition, paddies near forest edges are likely to receive and retain water from adjacent mountain slopes, resulting in relatively wet conditions (Natuhara 2021). Therefore, *P. p. brevipodus* may tend to utilize wet paddies located near forest edges as well.

In contrast, although drying of paddies during the non-irrigation period has also been considered a major driver of population decline in *P. nigromaculatus* (Uchiyama 2007), rainfall during this period may mitigate the drying of paddies in the study area. Therefore, drying may not be a primary limiting factor for the distribution of *P. nigromaculatus* in this region. Furthermore, in areas where the distributions of the two species overlap, morphological and ecological character displacement has been suggested to facilitate their coexistence (Ohtsuka and Mineta 2020). Thus, differences in the dependence of the two species on wet conditions may facilitate their spatial differentiation and coexistence along the wetness gradient.

Contribution of Unmeasured Factors and Interspecific interactions

We introduced a latent variable in the GLLVM to evaluate species associations that could not be explained by environmental variables, spatial autocorrelation, or river basin effects. The residual correlation between the two species was low ($r = 0.076$), providing little evidence for strong competitive or facilitative interactions. This result suggests that the distribution patterns of the two species were largely explained by the environmental variables considered in this study. However, the slightly positive residual correlation indicates that unmeasured environmental factors may have influenced the distributions of both species in a similar manner (Katayama et al. 2020).

Conservation implications

Although it has long been recognized that species diversity increases in heterogeneous landscapes with diverse habitats and environmental conditions (Estrada-Carmona et al., 2022; Stein et al., 2014; Uematsu et al., 2010), our study demonstrates that heterogeneity in topographic wetness, an environmental factor not readily visible from the surface, also plays an important role in maintaining biodiversity in agricultural landscapes. Differences in wet conditions have been reported to promote niche partitioning among wetland plants and animals (Araya et al. 2011; Higashikawa et al. 2019), suggesting that the importance of wetness heterogeneity revealed in this study may be broadly applicable to a wide range of wetland ecosystems including paddy fields. Therefore, the conservation and restoration of wetland landscapes

should focus not only on maintaining or recovering wetland area, but also on preserving diverse topological features, which create wetness heterogeneity at the landscape scale. Wetness heterogeneity may promote the coexistence of diverse wetland species by providing a variety of niches for species with different environmental preferences and thereby reducing interspecific competition.

Declarations

Competing Interests

Toshifumi Minamoto is an inventor of a patent for the use of BAC for eDNA preservation.

Author Contribution

A.O. conceived the research idea. A.O., A.U., and T.M. designed the study. A.O. and H.T. collected environmental samples. A.O. performed the experiments and data analyses. T.M. supervised the research. A.O. and T.M. wrote the draft of manuscript. All authors reviewed and approved the final manuscript.

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Data Availability

The datasets generated and/or analysed during the current study will be made publicly available in an appropriate repository upon acceptance of the manuscript. Repository information and a persistent identifier (DOI or accession number) will be provided after deposition.

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Figures

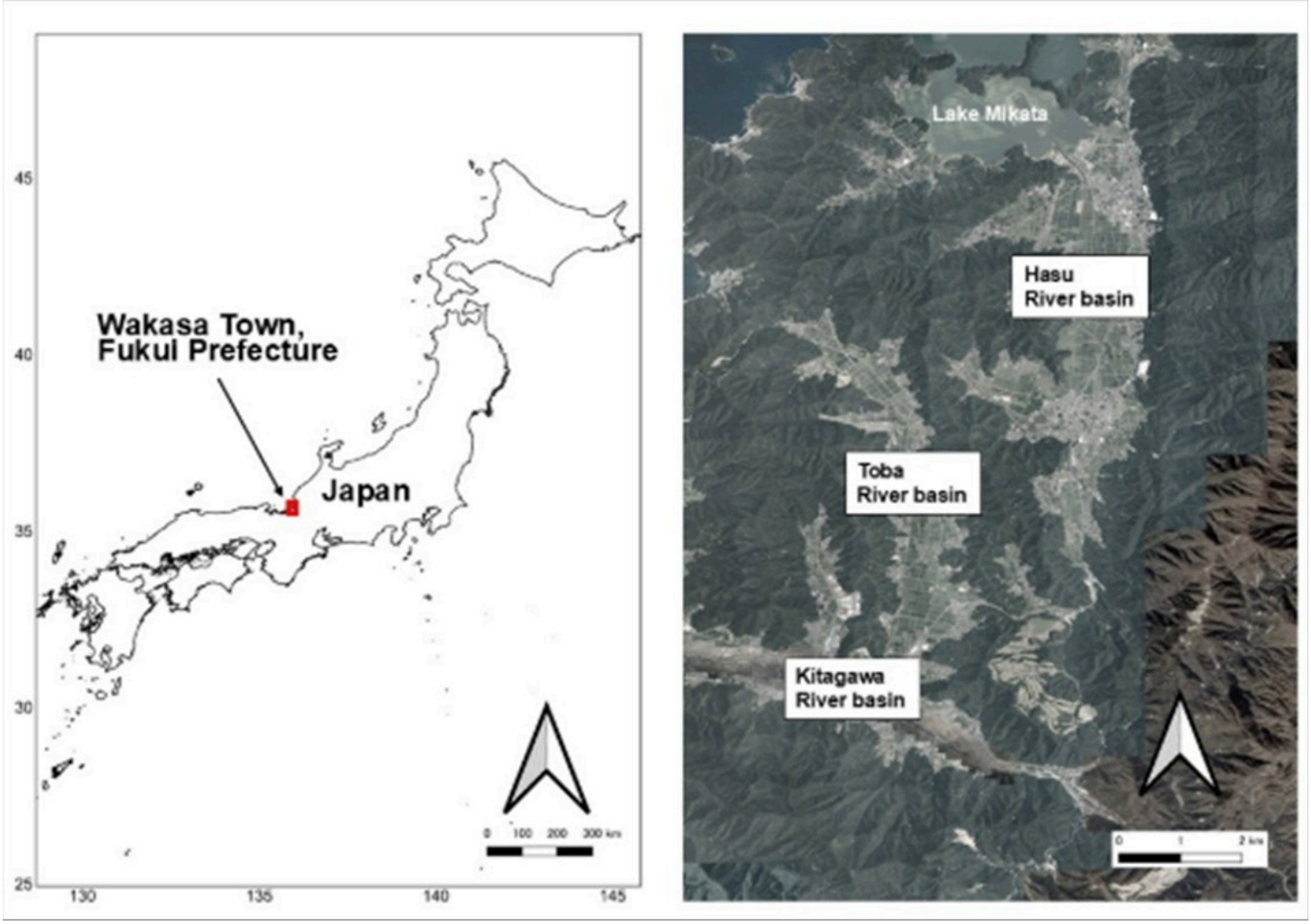


Figure 1

Study area. The study area is Wakasa Town in Fukui Prefecture, located in the Hokuriku region of Japan. The numbers on the axes of the Japan map indicate longitude (East longitude) and latitude (North latitude).

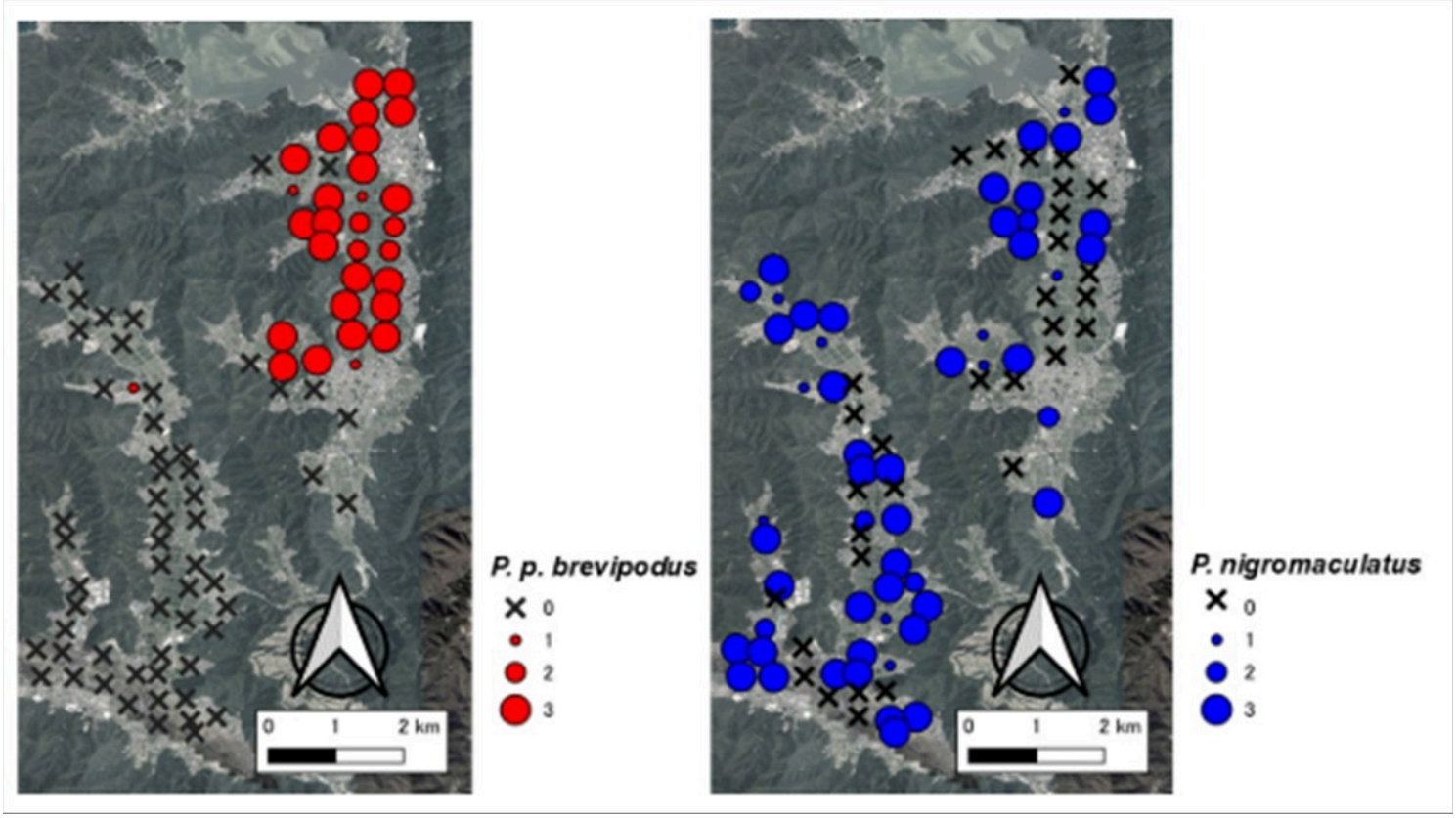


Figure 2

Results of the environmental DNA distribution survey of *Pelophylax porosus brevipodus* and *Pelophylax nigromaculatus* based on PCR analysis. The left panel shows the results for *P. p. brevipodus* and the right panel shows the results for *P. nigromaculatus*. Circles indicate sites where the DNA of the target species was detected by PCR, and the size of each circle represents the number of positive detections out of three PCR replicates. Crosses indicate sites where the target species DNA was not detected.

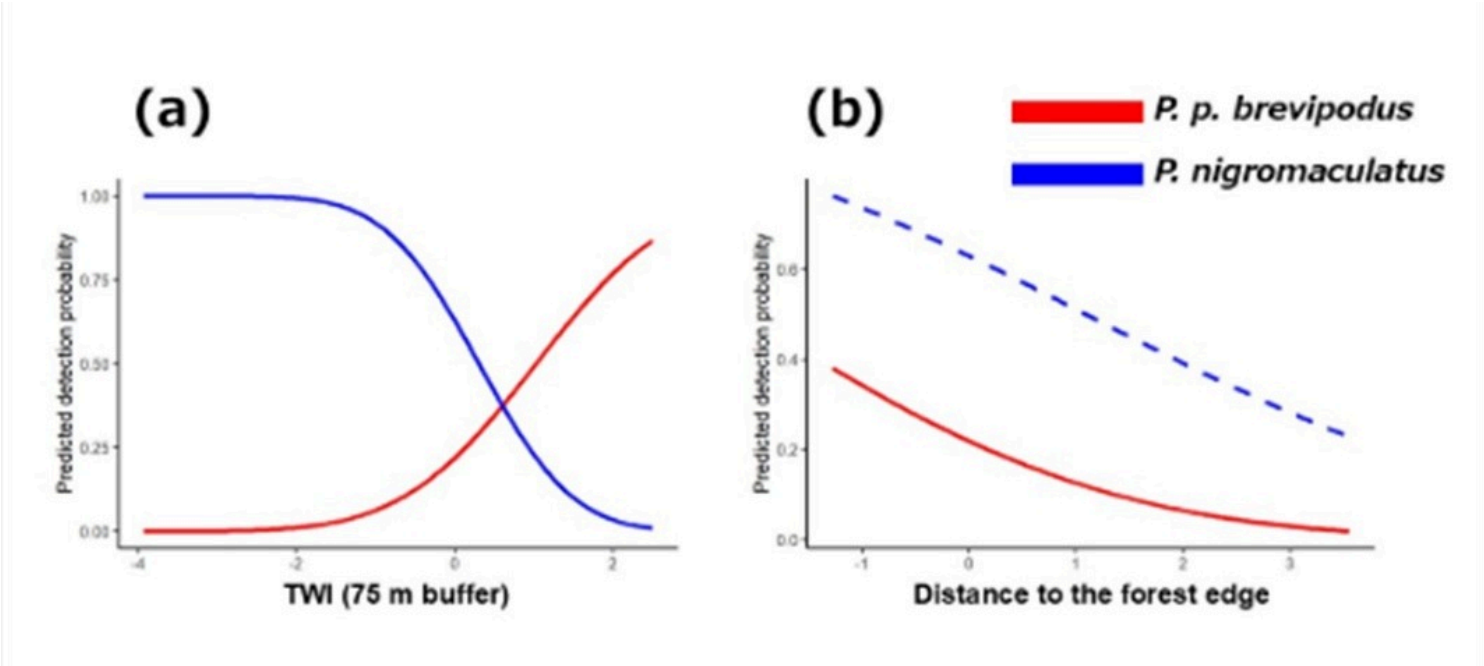


Figure 3

Predicted environmental DNA (eDNA) detection probabilities of *Pelophylax porosus brevipodus* and *Pelophylax nigromaculatus* in relation to environmental variables. The horizontal axis represents each environmental variable, and the vertical axis shows the predicted eDNA detection probability of the two frog species. The red lines indicate *P. p. brevipodus*, and the blue lines indicate *P. nigromaculatus*. Solid lines represent significant relationships between eDNA detection probability and environmental variables, whereas dashed lines indicate non-singificant relationships between them.

Supplementary Files

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- Appendix.docx