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Marine and Freshwater Research

Supplementary Material

Environmental DNA metabarcoding reveals the effect of river slope on diadromous fish communities in island rivers

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Supplementary methods

Detailed procedure of eDNA extraction and PCR

eDNA extraction and PCR were performed following Miya *et al*. (2022). eDNA was extracted from the cartridges using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The extracted DNA underwent a two-step PCR, followed by sequencing using the Miseq platform (Illumina, San Diego, CA, USA). In the first PCR, three universal primers (MiFish-U/E/U2) were used to amplify the hypervariable region of the mitochondrial 12S rRNA gene with eight technical replicates per sample. The products from these replicates were aggregated at equal volumes for each sample. Subsequently, they were purified, quantified, and diluted to 0.1 ng μL^{-1} with Milli Q water. Dual index sequences and flow cell binding sites for the Miseq platform were appended to these products, which underwent further amplification in the second PCR. During these processes, extraction blanks and PCR blanks for each step were prepared. The indexed libraries were electrophoresed on an agarose gel after being pooled at an equal volume to isolate target amplicons of ~≤370 bp. After measuring the concentration of the size-selected amplicons, they were diluted to 10.5 pM and were sequenced on a MiSeq platform using a MiSeq v2 Reagent Kit for 2 × 150-bp PE (Illumina) following the manufacturer's instructions.

Handling of the data of species detected in the extraction blanks

In the present study, following Zhu *et al*. (2023), filtration blanks, extraction blanks, and PCR blanks were prepared to check for contamination during the water sampling, on-site filtering, and eDNA extraction, and each step of the two-step PCR respectively. No eDNA reads of the 17 diadromous fish species that were presumed to inhabit the rivers on Yakushima Island (Table S1) were detected in any of the filtration and PCR blanks. No eDNA reads of the 17 species were detected in the extraction blanks, except for one species, *Rhinogobius brunneus*. The reads of this species were detected in all water samples collected; however, we did not exclude this species from the analysis. This is because the number of reads amplified from the extraction blanks was minimal (four reads) and considerably fewer than those amplified from the water samples collected at all stations (Table S1). Additionally, this species is a common species found in numerous rivers on this island (Motomura and Harazaki 2017), further justifying its inclusion in the analysis.

Table S1. Number of *Rhinogobius brunneus* reads amplified from the water samples collected from each station.

Station	Number of reads
GKN	21593
ISO1	5153
ISO2	14796
ISO3	260023
MYN1	18880
MYN2	13557
NGT	6570
STK	19374
JON	9705
TAB	33674
ANB1	5563
ANB2	5449
SUZ	43770
NKM	15001
KRO	8025
OKW1	27105
OKW2	6650

Details of preliminary analysis

In the present study, we assumed that the diadromous fish species detected in the eDNA sample collected from the lowest reach of each river represented all diadromous fish species inhabiting that river. This decision was based on the preliminary analysis described below, which indicated that the water sample collected from the lowest reach contained the eDNA of fish species inhabiting the lowest reach as well as eDNA of those inhabiting further upstream reaches in each river.

The initial objective of this study was to explore the relationship between the diadromous fish community and the riverine reach morphology at each station. Therefore, we classified the 17 water sampling stations into the following 4 morphological groups, following a modified rule from Bisson *et al*. (2006): 1. ISO2, ISO3, GKN, SUZ and OKW2: step-pool reaches, which are typical of high gradient reaches (2–8% gradient) and are characterised by a series of longitudinal steps alternating with pools; 2. MYN2, TAB and OKW1: pool-riffle reaches, which occur in low to moderate gradient reaches (1–2% gradient) and are characterised by a sequence of pools and riffles; 3. STK, JON and NKM: tidal reaches, which are influenced by the tide, and are characterised by gentle currents and relatively deep water depths during the high tide, but are similar to pool-riffle reaches during the low tide; and 4. NGT, ISO1, MYN1, ANB1, ANB2 and KRO: Estuarine reaches, which are always saline and are characterised by gentle currents and relatively monotonous and deep water depths regardless of the tides. Additionally, the Jaccard distances between each station were calculated from the presence or absence data of diadromous species at all stations as described in the Materials and Methods section. Hierarchical clustering was performed using the hclust function of the *stats* package (ver. 4.2.2) of the *R* software (ver. 4.2.2, R Foundation for Statistical Computing, Vienna, Austria, see https://www.R-project.org/) with the Jaccard distances and the ward method specified by "ward.D2".

The clustering analysis resulted in the stations being grouped into three clusters, namely clusters A, B and C (Fig. S1). Cluster A mainly comprised step-pool reaches, Cluster B was predominantly composed of pool-riffle and tidal reaches, and Cluster C consisted mainly of estuarine reaches. This clustering suggests that the reach morphology may influence the diadromous fish community within the reach.

However, the Venn diagram showing diadromous fish species detected in each cluster revealed an interesting pattern: all species detected in stations classified within Cluster A were also detected in stations classified within Cluster B, and likewise, all species detected in Cluster B stations were also found in Cluster C stations (Fig. S2). Step-pool reaches, which dominate cluster A, are typically found in upstream reaches with steep gradients (Bisson *et al*. 2022). Pool-riffle and tidal reaches, which predominate Cluster B, are generally found in downstream reaches compared to Step-pool reaches. Furthermore, estuarine reaches, which dominate Cluster C, are usually found in the most downstream sections of rivers. These results suggest that the diadromous fish species detected at each station do not solely reflect the species inhabiting that specific reach. Rather, the species inhabiting further upstream reaches were also detected. eDNA can be transported and detected over several kilometres, and in some instances, even more than 100 kilometres downstream of the DNA source (Pont *et al*. 2018). The steepness of the rivers in our study location may enhance this transport process.

This possibility is supported by the fact that in rivers where waters were collected from multiple sampling stations, all species detected in more upstream stations were also detected in more downstream stations (Table S1). Furthermore, species typically associated with middle to upper reaches and are unlikely to inhabit estuarine and lower reaches, such as *Rhinogobius brunneus* and *Sicyopterus japonicus* (Hosoya *et al*. 2019), were also detected in the water samples collected from estuarine and lower reaches (Table S1). Notably, the

spawning season of these gobies occurs from May to July in Japan for *R. brunneus* (Hosoya *et al*. 2019) and July to September for *Sicyopterus japonicus* (Iida *et al*. 2013). Given that our sampling was conducted in mid-April, eDNA from their larvae would not have contributed to their detection in estuarine and lower reaches. Based on these findings, we concluded that the diadromous fish species detected in the water sample collected from the lowest reach of each river reflect all diadromous fish species inhabiting that river.

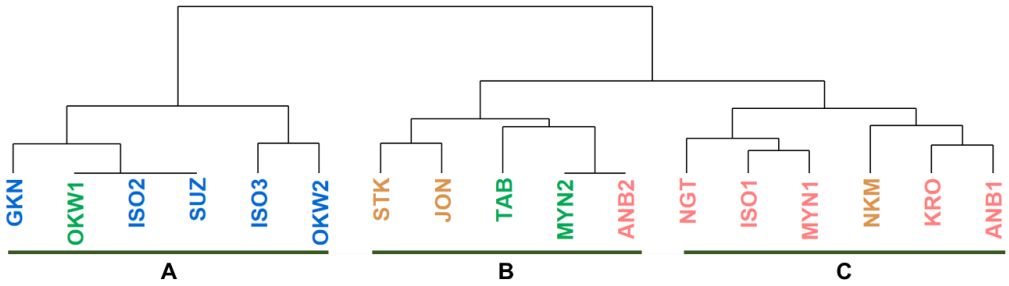

Fig. S1. Result of the hierarchical clustering. Blue, green, orange and red labels represent the step-pool, pool-riffle, tidal and estuarine reaches respectively. A, B and C indicate the three primary clusters.

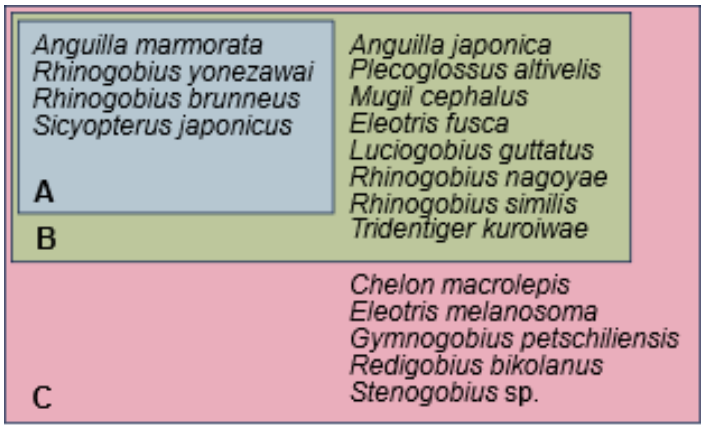

Fig. S2. Venn diagram showing the diadromous fish species detected in at least one station of each of the three clusters.

Table S2. Property of sampling river, station and number of detected fish species.

River group	River	Station	Reach	Distance from river mouth (km)	Number of species detected	Number of diadromous species detected	Slope (%) 1 km	Slope (%) 2 km
Estuarine	Nagata	NGT	Estuarine	0.8	16	15	0.2	0.9
	Isso	ISO1	Estuarine	0.6	19	14	0.2	0.4
		ISO2	Upper	3.6	5	5		
		ISO3	Upper	4.9	3	3		
	Miyanoura	MYN1	Estuarine	1.2	22	15	0.7	0.4
		MYN2	Middle	3.8	7	7		
	Anbo	ANB1	Estuarine	1.1	16	10	0.2	0.6
		ANB2	Estuarine	1.8	10	7		
	Kurio	KRO	Estuarine	1.4	16	13	0.0	0.1
Tidal	Shitoko	STK	Lower	0.3	10	10	3.1	8.6
	Jono	JON	Lower	0.1	12	11	7.0	7.8
	Tabu	TAB	Lower	0.2	10	8	4.0	4.8
	Nakama	NKM	Lower	0.3	9	9	5.3	6.1
Freshwater	Gakuno	GKN	Lower	0.1	4	4	11.2	12.1
	Suzu	SUZ	Upper	0.7	5	5	7.1	10.6
	Okawa	OKW1	Middle	0.3	5	5	15.9	10.9
		OKW2	Upper	0.8	4	4		

River group indicates a categorisation of rivers based on their estuary size (see Materials and methods section). Slope categories 1 and 2 km indicate the river slope calculated at 1 and 2 km from the river mouth.

Table S3. List of fish species whose taxon assignments were revised using neighbor-joining (NJ) trees and their occurrence records at the study site (Motomura and Harazaki 2017) among the 17 diadromous fish species used in the analysis.

Species	Detail of taxon revision
Tridentiger kuroiwae	Although this species cannot be distinguished from *Tridentiger obscurus* and *Tridentiger brevispinis* by the 12S rRNA gene sequence, this is the only species that has been found from this island.
Luciogobius guttatus	Although this species cannot be distinguished from *Luciogobius ryukyuensis* by the 12S rRNA gene sequence, this is the only species found on this island.

Table S4. Property of sampling stations and diadromous fish species detected at each station.

Station ID (Reach)	NGT (Estuarine)	ISO1 (Estuarine)	ISO2 (Upper)	ISO3 (Upper)	MYN1 (Estuarine)	MYN2 (Middle)	ANB1 (Estuarine)	ANB2 (Lower)	KRO (Estuarine)	STK (Lower)	JON (Lower)	TAB (Lower)	GKN (Lower)	SUZ (Upper)	NKM (Lower)	OKW1 (Middle)	OKW2 (Upper)	Total
Distance from river mouth (km)	0.8	0.6	3.6	4.9	1.2	3.8	1.1	1.8	1.4	0.3	0.1	0.2	0.1	0.7	0.3	0.3	0.8	
Species																		
Anguilla japonica	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	3
Anguilla marmorata	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	16
Plecoglossus altivelis	1	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	4
Chelon macrolepis	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	3
Mugil cephalus	1	1	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	7
Eleotris fusca	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	3
Eleotris melanosoma	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2
Gymnogobius petschiliensis	1	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	5
Luciogobius guttatus	1	1	0	0	1	0	0	0	0	1	1	1	0	0	1	0	0	7
Redigobius bikolanus	1	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	5
Rhinogobius nagoyae	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	4
Rhinogobius similis	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	0	0	11
Rhinogobius yonezawai	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	15
Sicyopterus japonicus	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	15
Stenogobius sp.	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
Tridentiger kuroiwae	1	1	0	0	1	1	1	1	1	1	1	0	0	0	1	0	0	10
Rhinogobius brunneus	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	17
Total number of diadromous species	14	13	4	2	14	6	9	6	12	9	10	7	3	4	8	4	3	-

Species numbers 0 and 1 indicates that the species either undetected or detected at each station.

Table S5. List of possible false-positive detections assessed based on the habitat use of the species (Hosoya *et al*. 2019).

Station	Detected species	Reason
ISO2	*Mugil cephalus cephalus*	Not found in upstream reaches
ISO3	*Rhinogobius nagoyae*	Not found in upstream reaches
SUZ	*Mugil cephalus cephalus*	Not found in upstream reaches
OKW2	*Rhinogobius nagoyae*	Not found in upstream reaches

Table S6. Results of generalised linear models (GLMs) showing the relationship between the number of diadromous fish species detected within each river and river slope, which was calculated at 1 and 2 km from the river mouth.

Riverine slope calculation	Term	Estimate ± s.d.	z-value	*P* value
At 1 km from river mouth	Intercept	2.53±0.12	20.23	<0.001
	Slope	-0.09±0.03	-2.70	0.0069
At 2 km from river mouth	Intercept	2.55±0.13	19.65	<0.001
	Slope	-0.07±0.03	-2.67	0.0076

Table S7. Results of linear models (LMs) showing the relationship between the Jaccard distance between rivers and the differences in river slopes between those rivers, which was calculated at 1 and 2 km from the river mouth.

Riverine slope calculation	r^2	Term	Estimate ± s.e.	*t*-value	*P*-value
At 1 km from river mouth	0.49	Intercept	0.34±0.03	12.73	<0.001
		Slope	0.03±0.005	6.46	<0.001
At 2 km from river mouth	0.41	Intercept	0.34±0.03	11.16	<0.001
		Slope	0.03±0.005	5.46	<0.001

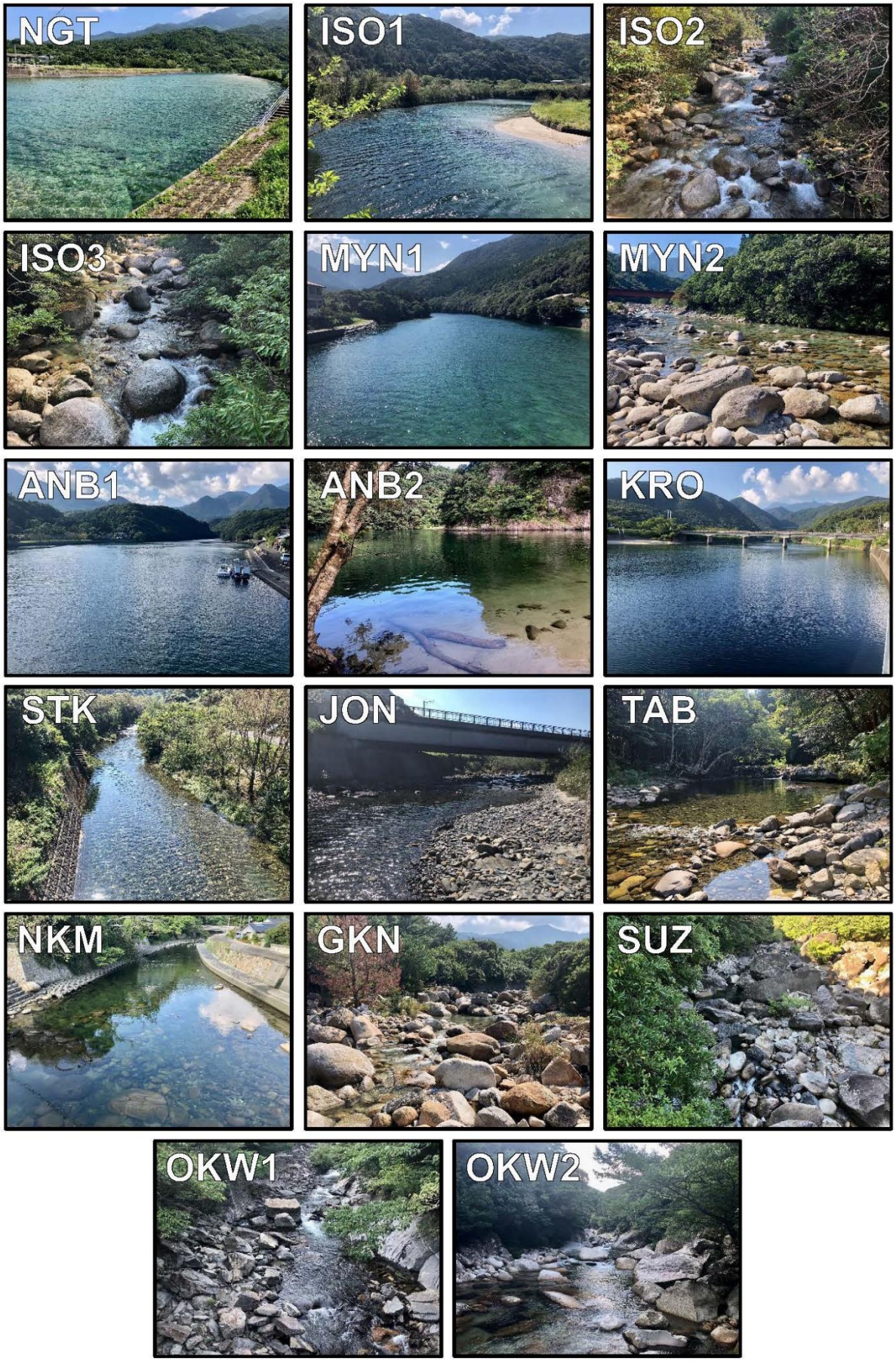

Fig. S3. Photograph showing the landscape of each sampling station. For unabbreviated river names, see Table S2.

Fig. S4. Photograph of the 88-m-high Oko Waterfall in the Okawa River.

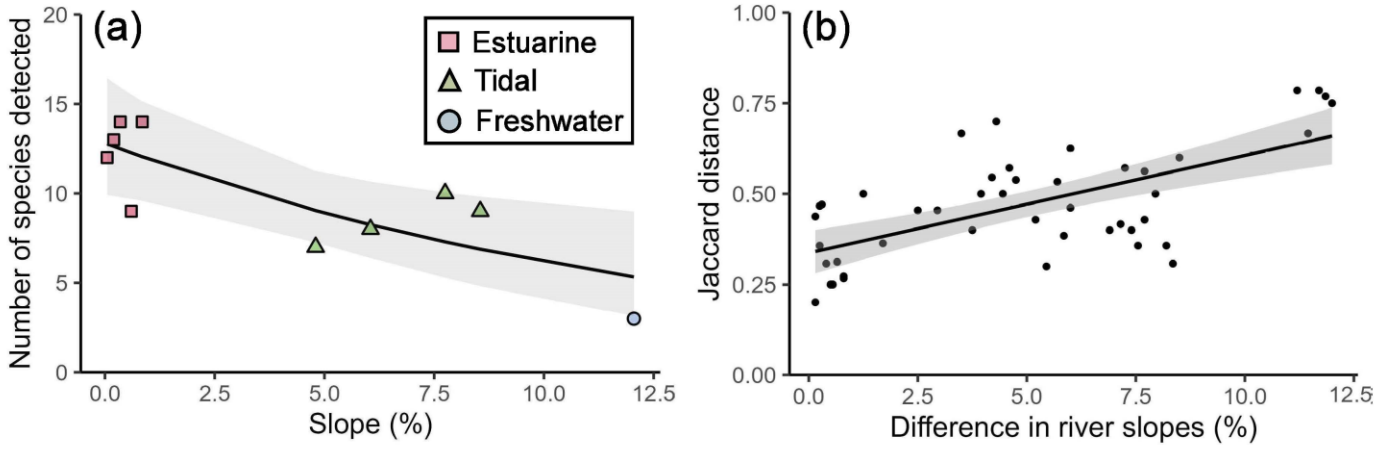

Fig. S5. (a) Generalised linear model (GLM) results showing the relationships between the number of diadromous species detected in each river and the river slope calculated at 2 km from the river mouth. Different symbols represent river groups categorised based on the estuary size. (b) Liner model (LM) results showing the relationship between the Jaccard distances between rivers calculated based on the presence or absence data of diadromous fish species and the differences in river slopes between those rivers.

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