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Two distinct genetic lineages of *Drunella trispina* (Uéno, 1928) (Ephemeroptera: Ephemerellidae) cohabit but are reproductively isolated in Kyoto, Japan

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ABSTRACT

Based on the analyses of mitochondrial cytochrome *c* oxidase subunit I (*cox1*) and nuclear 18S rRNA genes, we found two distinct genetic lineages of *Drunella trispina* (Uéno, 1928), that are morphologically indistinguishable at the nymphal stage. These two groups seem to have different habitat niches: one prefers the uppermost reaches, of which Strahler's stream order is three or less, and the other inhabits relatively lower reaches with a stream order of three or larger. However, they coexist in Kibune Stream (called Kibunegawa), a tributary of the Kamo River, in Kyoto, central Honshu, Japan. The sampling site of Kibunegawa originally had a mountain–stream environment with step-pool configuration (at the beginning of the third order). Over the past 60 years, several small check dams to prevent sediment erosion have been constructed along Kibunegawa, resulting in the formation of larger pools and slow riffles, and the emergence of various secondary microhabitats. The results of the DNA analyses indicated that the two genetic groups in Kibunegawa rarely interbred. Preliminary observation of egg surface morphology suggested a potential diagnosis to distinguish between these two distinct genetic groups.

KEYWORDS

cox1; habitat preference; rRNA; speciation

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Introduction

The spiny crawler mayfly *Drunella trispina* (Uéno, 1928) is one of the most commonly observed taxa in the upstream reaches of streams and geographically widespread in Japan (Ishiwata 2000; Ishiwata 2002; Tamura and Kagaya 2016; Jo and Tojo 2019; Wakimura et al. 2024). The final-stage nymphs of this species emerge from mid-May to June in central Honshu, Japan (Tamura and Kagaya 2016, 2017).

In the course of DNA barcoding of mayflies, Wakimura et al. (2020) have preliminarily noted that *D. trispina* specimens obtained from two localities conformed to two distinct genetic lineages. Wakimura et al. (2024) investigated the cytochrome *c* oxidase subunit I (*cox1*) molecular phylogeny of the genus *Drunella* Needham, 1905 inhabiting Japan and found that two distinct mitochondrial haplogroups of *D. trispina* were distantly related, comparable to the distances between these two groups and *Drunella triacantha* (Tshernova, 1949). One mitochondrial haplogroup of *D. trispina* was sampled from Nagano (Jo and Tojo 2019), Nara, Wakayama, and Kyoto Prefectures (designated as Clade 1 in Wakimura et al. 2024), and the other haplogroup was sampled from Kanagawa, Kyoto, and Okayama Prefectures (designated as Clade 2 in Wakimura et al. 2024). As for the samples collected on 22 April 2023 and before, the specimens belonging to Clade 1 (hereafter designated as Cl. 1) were obtained from the lower reaches of mountain streams, and those belonging to Clade 2 (hereafter designated as Cl. 2) were from the upper reaches of mountain streams (Table 1). These haplogroups were observed in the tributaries of Kamo River System, Kyoto Prefecture; the Kumogahata Stream (called Kumogahatagawa) habitat retained only one haplogroup (Cl. 1) and Kibunegawa habitat maintained both Cl. 1 and Cl. 2 haplogroups. These two genetically distinct groups, however, were not morphologically distinguishable at the nymphal stage. They possess three frontal spines on the head: one median straight and the other two laterally curved inward. The inner margin of the femur of the foreleg is armed with strong teeth of unequal size, as described by

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Uéno (1928). These characteristics were shared by all specimens regardless of the mitochondrial haplogroups.

In this study, we have analysed partial coding regions of the mitochondrial *cox1* and the nuclear 18S rRNA genes of *D. trispina* nymphs in Kamo River System more extensively, to elucidate the genetic structure of *D. trispina* populations in Kumogahatagawa and Kibunegawa.

Material and methods

Sampling

The specimens examined in this study were sampled from Kanagawa, Nara, Wakayama, Shiga, Kyoto, and Okayama Prefectures, central Honshu Island, Japan (Figure 1), and Table 1 lists the sampling sites in detail. The examined specimens are listed in Supplementary Table S1 (collected on 22 April 2023 or before) and Table S2 (collected on 28 April 2023 or after). Specimens (nymphs) were collected from a riverbed by kicking or hand-picking methods and immersed in absolute ethanol immediately after sampling, except for those that were reared in the laboratory. Samples were preserved in absolute ethanol and stored at ambient temperature until further examination.

[Figure 1 and Table 1 near here]

Allometric measurements

Specimens collected from Kumogahatagawa and Kibunegawa in 2023 were photographed under the microscope model SZ16 (Olympus, Tokyo, Japan), and their body sizes were measured on the photographs using ImageJ version 1.53e (http://imagej.nih.gov/ij). The width of the mesothorax and the length between the tip of the wing bud and the fore-end of the mesothorax (average of the right and left) were defined as shown in Figure 2.

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[Figure 2 near here]

DNA analyses

Total genomic DNA was isolated from the leg muscles of the specimens by treatment with proteinase K (Promega, Madison, WI, USA), followed by phenol extraction and precipitation with ethanol, as previously described (Wakimura et al. 2020). Target regions for DNA sequencing were amplified by PCR using GoTaq Green Master Mix (Promega, Madison, WI, USA), with the primer pairs LCO1490 and HCO2198 (Folmer, Black, Hoeh, Lutz, and Vrijenhoek 1994) for *cox1*, and KOBO18SF1 and KOBO18SR1 (Wakimura et al. 2016) for 18S rRNA gene partial sequences. The amplified DNA fragments were subjected to DNA sequencing using the BigDye terminator v3.1 and an ABI3730xl DNA Analyzer (Life Technologies, Carlsbad, CA, USA). The sequence data were deposited in the INSD International DNA Sequence Databases (GenBank/ENA/DDBJ), and their accession numbers are shown in the specimen list (Supplementary Tables S1 and S2). Pairwise distances of *cox1* sequences were calculated by the program ‘dnadist’ in PHYLIP (Felsenstein 1980) with F84 substitution model (Felsenstein and Churchill 1996). *Cox1*-based phylogram of *D. trispina* was constructed using PhyML 3.0 (Guindon et al. 2010) with the Smart Model Selection option (Lefort, Longueville, and Gascuel 2017). The tree was visualised using FigTree ver. 1.4.4 (Rambaut 2018).

Results

Drunella trispina is divided into two distinct genetic lineages

The maximum likelihood phylogram of 234 specimens of *D. trispina* is shown in

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Supplementary Figure S1. As noted previously, *cox1* partial coding sequences of *D. trispina* were grouped into two clades; Cl. 1 and Cl. 2, based on sequence similarity. Average genetic distances within the Cl. 1 haplogroup (140 specimens in total) and the Cl. 2 haplogroup (94 specimens in total) were 0.0068 and 0.0040, respectively, while average genetic distance between the Cl. 1 and Cl. 2 hapologroups of *D. trispina* was 0.1478. Those between a *cox1* sequence of *Drunella basalis* (Imanishi, 1937) (OP18492) and each of *D. trispina* Cl. 1 and Cl. 2 were 0.2072 and 0.2032, respectively. Those between a *cox1* sequence of *D. triacantha* (OP901906), and each of *D. trispina* Cl. 1 and Cl. 2 were 0.1815 and 0.1758, respectively.

We also examined the partial sequences of 18S rRNA and found a single nucleotide polymorphism (SNP) within the 18S rRNA region (see Appendix 4, supplementary file). The nucleotide position of 104th in the PCR-amplified fragment of KOBO18SF1-KOBO18SR1 contained adenosine (104A) or guanosine (104G). These genotypes are shown in the species list (Supplementary Tables S1 and S2).

The Cl. 1 mitochondrial haplogroup was sampled from Nara, Wakayama, Shiga, and Kyoto Prefectures, and the Cl. 2 haplogroup was sampled from the Kanagawa, Kyoto, and Okayama Prefectures (Table 1). The Cl. 2 haplogroup seems to prefer the uppermost reaches, whereas the Cl. 1 haplogroup inhabited the relatively lower reaches, as shown by Strahler's stream order (Strahler 1957) and the river morphology types defined by Kani (1944) and Rosgen (1994) (Table 1). Habitat segregation was evident between the Cl. 1 and Cl. 2 mitochondrial haplogroups that were identified at all sampling sites except Kibunegawa in Kyoto Prefecture.

All individuals belonging to the Cl. 1 haplogroup had 104G in 18S rRNA. Individuals belonging to the Cl. 2 haplogroup (92 out of 94 specimens) had 104A in 18S rRNA, but two exceptions had the Cl. 2-104G genotype sampled from Kibunegawa in 2023.

Allometry of Drunella trispina nymphs collected in Kumogahatagawa and Kibunegawa in

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2023

To evaluate the developmental status of the two genetic groups, allometric relationships in the body size of nymphs sampled from Kumogahatagawa and Kibunegawa in April and May 2023 were examined. The results are listed in the rightmost columns of Supplementary Table S2 and represented in the scatter plots (Figure 3). Body size allometry clearly discriminated between the final instar and the penultimate stages (panel C of Figure 3). In the panel A of Figure 3, the allometric distribution of the specimens collected on 28 April shows that the nymphs in Kumogahatagawa were in the penultimate stage, whereas a few of those in Kibunegawa advanced to the final instar stage. This may be due to a slight difference in water temperature, as the Kibunegawa has an abundant supply of groundwater, while the Kumogahatagawa is affected by the cold in winter and early spring. Most specimens collected from both sampling sites in May were in the final instar stage (panel B of Figure 3). The genotypes (Cl. 1-104G, Cl. 2-104A, and Cl. 2-104G) of the Kibunegawa specimens are classified by colour in the panel D of Figure 3.

[Figure 3 near here]

Discussion

We characterized two genetic lineages of *D. trispina* that rarely interbred, with only two individuals (Cl. 2-104G) of the potential offspring of the hybrid (Cl. 2-104A × Cl. 1-104G) out of the 116 specimens (43 for Cl. 1-104G, 71 for Cl. 2-104A, and 2 for Cl. 2-104G) collected from Kibunegawa in 2023. The allometric measurement indicated that nymphal developmental stages in Kibunegawa were identical regardless of genotype (Figure 3D), implying the possibility of mating between genotypes, however. Genetically distinct populations may result

from parapatric speciation owing to habitat preferences. The reproductive isolation may have been established between the two genetic lineages of *D. trispina* with habitat segregation, as one (Cl. 2-104A) prefers the uppermost reaches with the river morphology type of Aa or Aa-Bb transition defined by Kani (1944), and the other (Cl. 1-104G) inhabits the relatively lower reaches of Aa-Bb transition or Bb type.

It is likely to hypothesize that the invasion of Cl. 1-104G population into Kibunegawa habitat has occurred, where Cl. 2-104A population was already present. Since the 1960s, several check dams have been constructed in Kibune Stream Basin to control sediment erosion. This caused the emergence of the Aa-Bb transition or Bb type reach (Figure 4), where Cl. 1-104G populations can inhabit an area. Further investigations are required to test this hypothesis, including sampling in the headwaters of Kibunegawa to determine the upper limit of Cl. 1-104G habitat. And also, sampling in Kibunegawa between Azodani-deai and the confluence with Kumogahatagawa (where only Cl. 1-104G genotype exists) will help to determine the lower limit of Cl. 2-104A habitat. The sampling sites at Kumogahatagawa and Kibunegawa in this study are geographically close (approximately 4.4 km apart; see Figure 5). Similar situation had been observed for a burrowing mayfly *Ephemera orientalis* McLachlan, 1875. Three species in the genus *Ephemera* Linnaeus, 1758 are known to be distributed in Japan, from upstream to downstream. *Ephemera japonica* McLachlan, 1875*, Ephemera strigata* Eaton, 1892, and *E. orientalis* inhabit Aa or Aa-Bb transition type reaches in mountain regions, Aa-Bb transition or Bb type reaches in mountain and open field, and Bb or Bc type reaches in lower open field (< 100 m in altitude), respectively according to the river morphology types defined by Kani (1944). *E. orientalis* occasionally inhabit above check dams in higher places (Watanabe 1985; Okamoto and Tojo 2021).

[Figures 4 and 5 near here]

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Egg morphology can be one of the crucial taxonomic identifiers of mayflies (Koss 1968; Koss and Edmunds 1974; Ubero-Pascal and Puig 2009). We preliminarily observed the eggs obtained from the specimen OPU_BS_2022-159-KM-EP having Cl. 1-104G genotype (Kato 2024) by scanning electron microscopy (SEM). The SEM image showed an ovoid form with single polar cap as a common feature for the genus *Drunella*. Certain details, however, were different from *D. trispina* egg that was previously reported in the plate 9-figure 8 of Ishiwata and Fujitani (2018). More examination of a number of egg samples will elucidate definite taxonomic characters in these clades. Although no DNA data were available for the source specimen of the SEM image shown in Ishiwata and Fujitani (2018), the locality of the specimen (Sakawa River in Kanagawa Prefecture) was close to the site, Fujikuma Stream (called Fujikumagawa) in Kanagawa Prefecture, where Cl. 2-104A genotype was sampled.

Conclusion

Two distinct genetic lineages of *Drunella trispina* have been characterized by mitochondrial *cox1* and nuclear 18S rRNA genes. These groups apparently show the different habitat preference; uppermost reaches (Cl. 2-104A genotype) or lower reaches (Cl. 1-104G genotype). The populations of both genotypes cohabit but rarely interbred in Kibunegawa Azodani-deai near to the headwaters, where lower-reach characters have been generated by the construction of check dams. Although the distributions of these genetic groups in Kibune Stream Basin are yet unclear, environmental modification by check dams may have influenced the habitat segregation of the species.

Disclosure statement

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The authors declare no potential conflict of interest associated with this manuscript.

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List of figure captions

Figure 1. Rough indication of the sampling sites on Honshu Island.

Drunella trispina (Uéno, 1928) specimens were collected from Kanagawa, Shiga, Kyoto, Nara, Wakayama, and Okayama Prefectures in central Honshu, Japan. Details of the sampling sites are listed in Table 1. The sampling sites of Kumogahatagawa and Kibunegawa are very close and indistinguishable on the map.

Figure 2. Definition of the body size measurement.

The distance between the fore-end edges of the mesothorax (indicated by blue arrows) is defined as “mesothorax width”. The average distance between the centre of the fore-end of the mesothorax (indicated by a white arrow) and tips of wing buds (left and right; indicated by red arrows) is used as “mesothorax-wing bud length”. An image of the OPU_BS_2023-021-SO-EP specimen is presented.

Figure 3. Allometry of *Drunella trispina* (Uéno, 1928) nymphs sampled in April 2023 and May 2023.

The body sizes of the specimens are shown as scatter plots, where the mesothorax-wing bud length is plotted against the mesothorax width. The left and right wings were measured and averaged. Panel A: Specimens sampled on 28-April-2023 at Kumogahatagawa (35°06'17"N, 135°43'53"E) and Kibunegawa (35°08'03"N, 135°45'50"E). Panel B: Specimens sampled on 13-May-2023 and 17-May-2023 in Kumogahatagawa and Kibunegawa. Panel C: Overlaid plot of panels A and B. The Kumogahatagawa and Kibunegawa specimens are shown with orange and light blue markers, respectively (Panels A–C). Panel D: Specimens sampled in April and May at Kibunegawa. The genotypes [Cl. 1-104G], [Cl. 2-104A], and [Cl. 2-104G] are indicated by orange, light blue, and black markers, respectively.

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Figure 4. Representative landscape of Kumogahatagawa and Kibunegawa sampling sites. Panel A: Kumogahatagawa sampling site (upstream view). Panel B: Kibunegawa sampling site at the confluence with Azodani (Azodani-deai). White arrows indicate the upstream directions of the streams. Panel C: Pool and gentle runs immediately after the Azodani-deai, which appeared because of the accumulation of sediment deposited by the check dam. Panel D: Dam body constructed in 1960. The images took on 18 February 2024.

Figure 5. Topographical map showing tributaries of Kamo River in Kyoto.
KH, Kumogahatagawa sampling site; KB, Kibunegawa Azodani-deai sampling site. Watercourses are indicated by curved blue lines. The distance scale bar (500 m) is shown at the bottom right. The map was adapted from the Geospatial Information Authority of Japan website (https://maps.gsi.go.jp/#14/35.109588/135.740719/).

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Appendix 1.

Supplementary Table S1. Specimens collected on or before 22-April-2023.

Appendix 2.

Supplementary Table S2. Specimens collected from Kumogahatagawa and Kibunegawa on or after 28-April-2023.

Appendix 3.

Supplementary Figure S1. Maximum likelihood phylogenetic tree of *cox1* in *Drunella trispina* (Uéno, 1928).

Cox1-based phylogram is reconstructed by PhyML 3.0 (Guindon et al. 2010) with the substitution model HKY85 +G selected by Smart Model Selection option (Lefort, Longueville, and Gascuel 2017). Scores of bootstrapping reproducibility (> 80%) were shown at the nodes. INSD accession numbers of *cox1* sequence data are listed in Supplementary Tables S1 and S2. The tree is rooted by *Drunella basalis* (Imanishi, 1937) (OP018942) as an outgroup, along with *Drunella triacantha* (Tshernova, 1949) (OP901906).

Appendix 4.

A fasta file of 18S rRNA partial sequences showing 104G and 104A SNPs.

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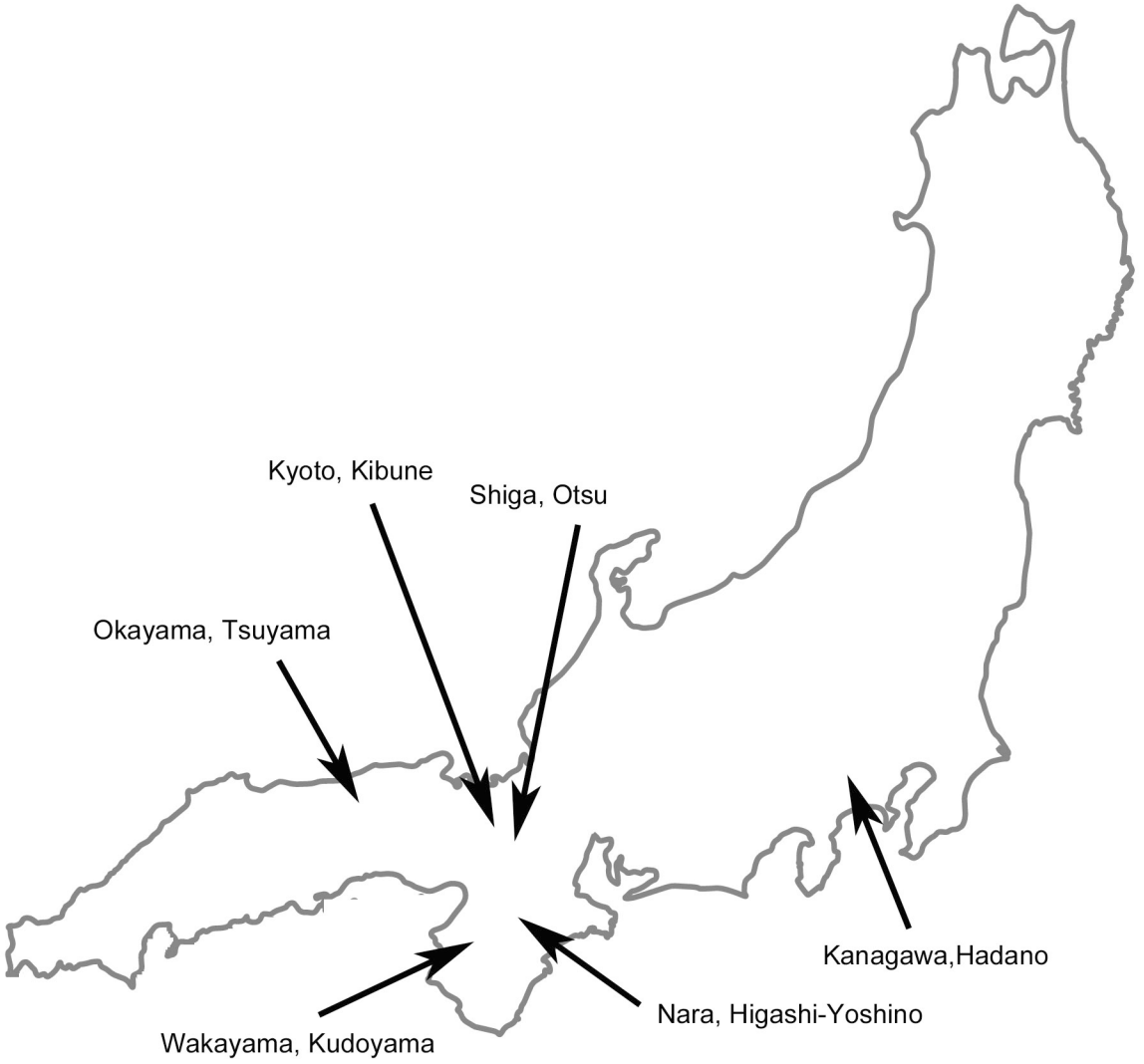
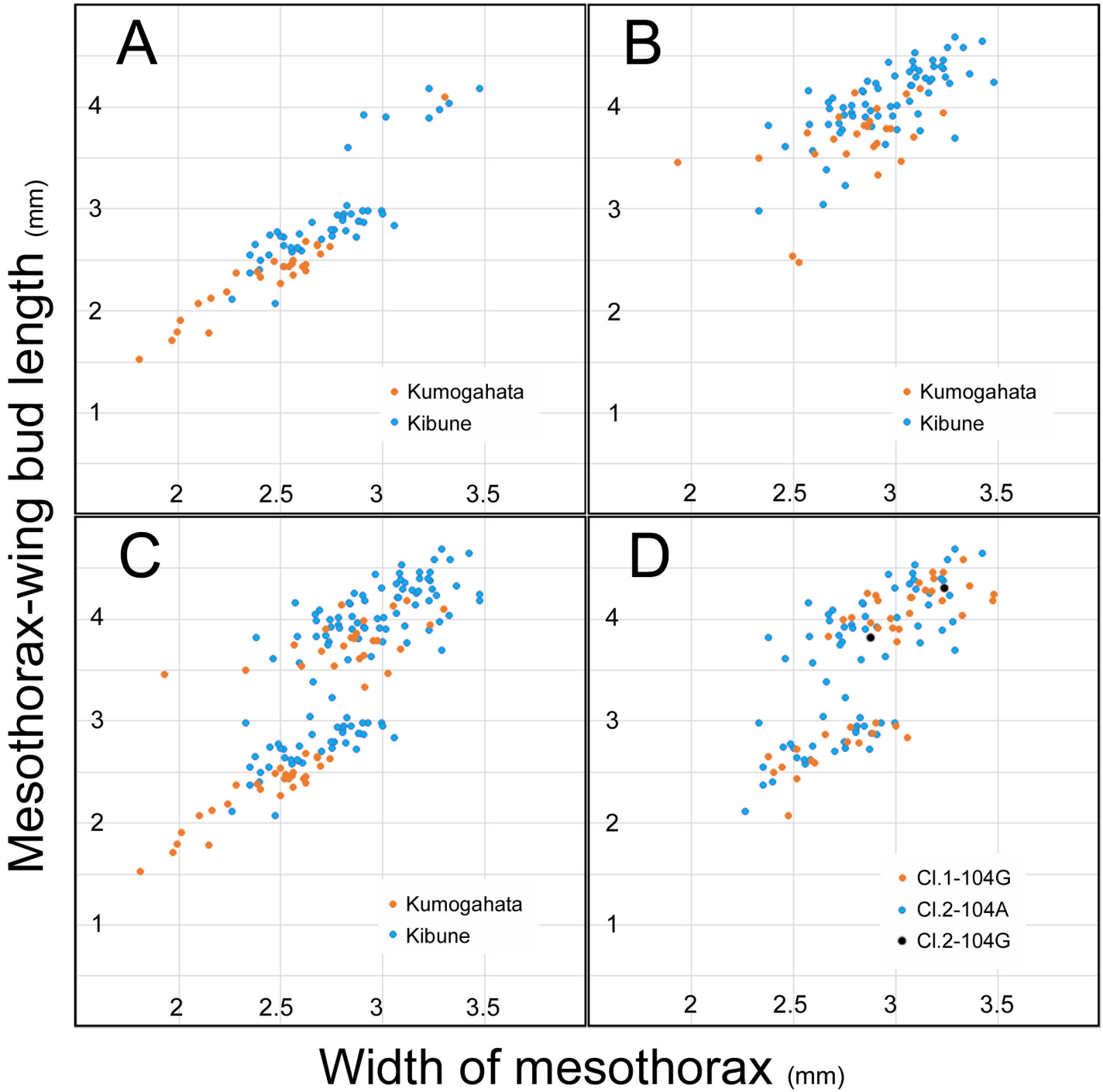
Table 1. Sampling locations of *Drunella trispina* (Uéno, 1928)

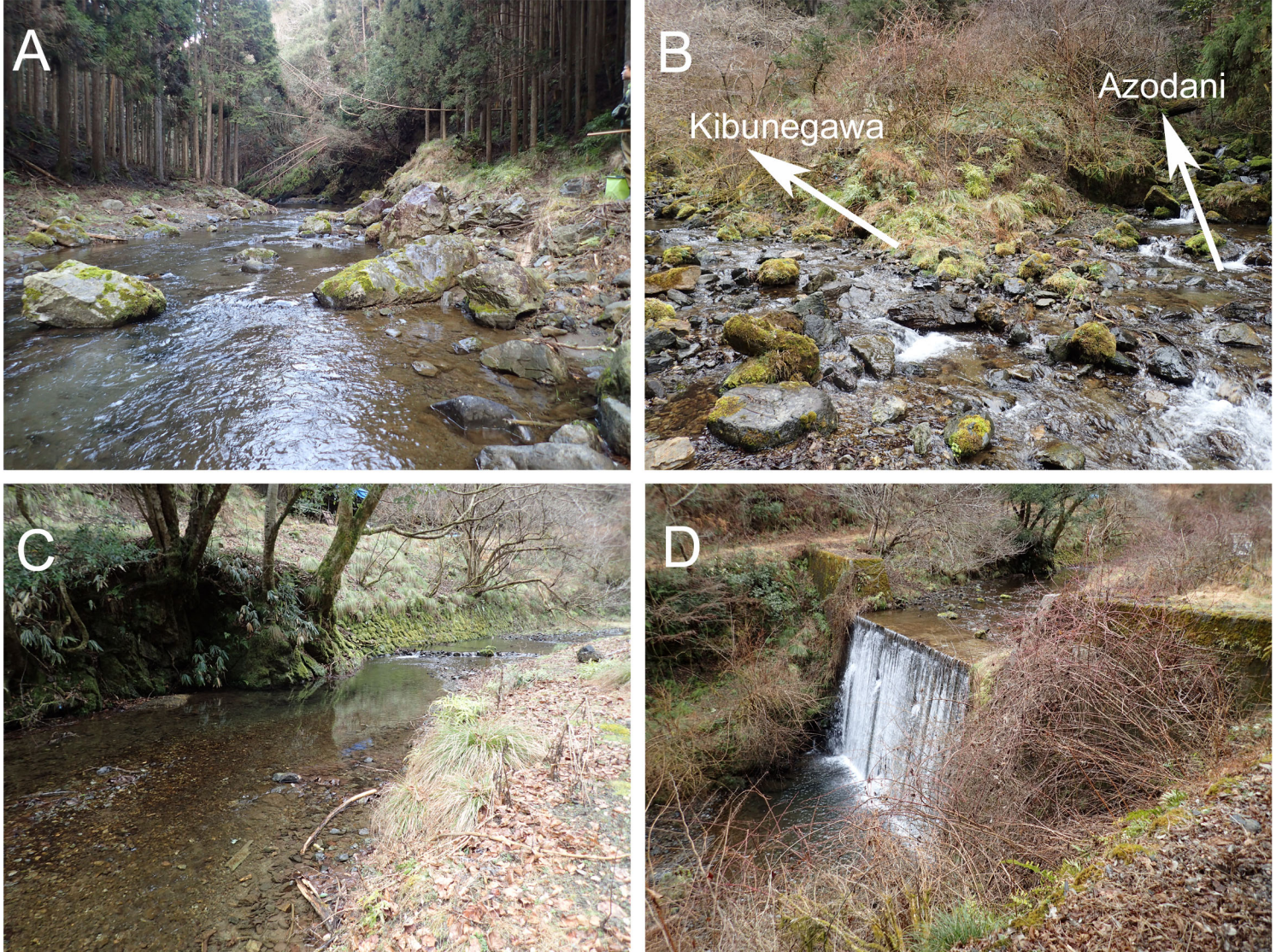
Sampling site	latitude-longitude	altitude (m)	Stream order	Stream morphology type		*cox1* haplogroup	
			Strahler (1957)	Kani (1944)	Rosgen (1994)	(number of samples[*1])	(number of samples[*2])
Kyoto, Takanogawa, Kochidani	35°08'11"N, 135°50'03"E	254	4	Bb	C	Cl. 1 (2)	
Kyoto, Kumogahatagawa, Omagari	35°06'17"N, 135°43'53"E	233	4	Bb	C	Cl. 1 (9)	Cl. 1 (53)
Shiga, Omiyagawa	35°04'25"N, 135°52'07"E	125	3	Aa-Bb	B	Cl. 1 (3)	
Nara, Takamigawa	34°23'53"N, 135°59'36"E	306	4	Bb	B	Cl. 1 (7)	
Wakayama, Kiinyugawa	34°16'42"N, 135°36'28"E	160	4	Bb	B	Cl. 1 (13)	
Wakayama, Kiinyugawa, Kitamata-deai	34°15'56"N, 135°38'35"E	248	4	Bb	C	Cl. 1(8)	
Kyoto, Kibunegawa, Azodani-deai	35°08'03"N, 135°45'50"E	382	beginning of 3	Aa-Bb	B	Cl. 1 (1) and Cl. 2 (13)	Cl. 1 (43) and Cl. 2 (73)
Kyoto, Kibunegawa, Yuyagadani-deai	35°07'51"N, 135°45'56"E	352	3	Aa	A	Cl. 1 (1) and Cl. 2 (3)	
Kanagawa, Fujikumagawa	35°26'05"N, 139°12'43"E	648	2	Aa-Bb	B	Cl. 2 (3)	
Okayama, Ochiaigawa	35°15'02"N, 134°07'52"E	592	3	Aa	A	Cl. 2 (2)	

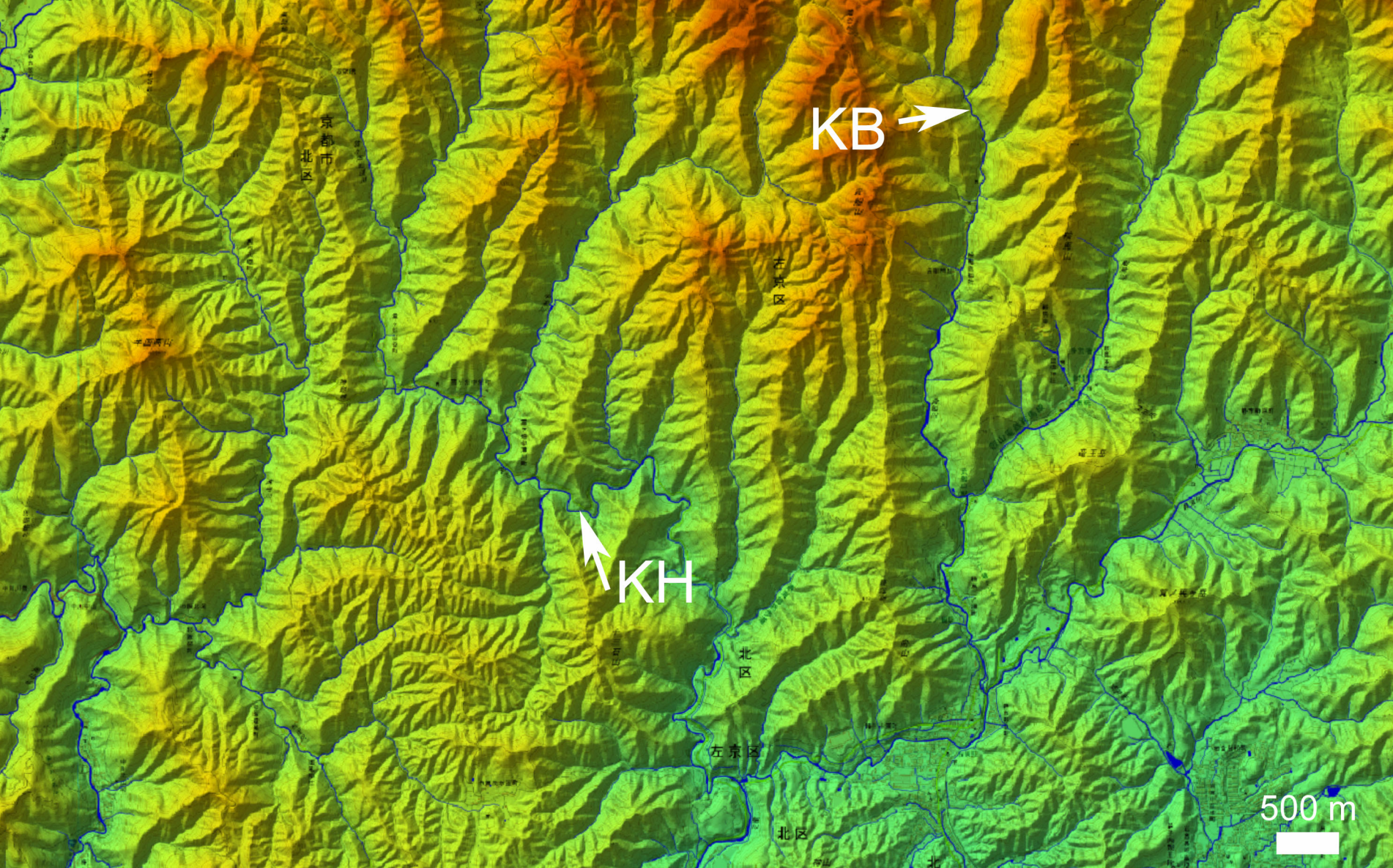
*[1]Samples collected by 22-April-2023 (left column)

*[2]Samples collected on 28-April-2023 and after (right column)


Kyoto, Kibune
Shiga, Otsu
Okayama, Tsuyama
Kanagawa,Hadano
Nara, Higashi-Yoshino
Wakayama, Kudoyama


A
B
C
D
Mesothorax-wing bud length (mm)
Width of mesothorax (mm)
Kumogahata
Kibune
Cl.1-104G
Cl.2-104A
Cl.2-104G
1
2
3
4
2
2.5
3
3.5


A
B
Kibunegawa
Azodani
C
D


KB
KH
京都市北区
右京区
北区
左京区
北区
500 m