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Microhabitat use by larvae of the endangered dragonfly *Sympetrum pedemontanum elatum* (Selys) in Japan

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ABSTRACT

Sympetrum pedemontanum (Müller in Allioni) (Odonata: Libellulidae), which is distributed widely in the Eurasian continent and its neighboring islands, is listed as a Least Concern species in the International Union for Conservation of Nature Red List (2015). In Japan, however, the population of its subspecies *S. pedemontanum elatum* (Selys) has been rapidly decreasing since the 1970s. In order to conserve this subspecies, it is important to understand the seasonal microhabitat use by its larvae. However, this has been a difficult task because larvae of *S. pedemontanum elatum* often coexist with those of a common congener, *S. eroticum* (Selys), and cannot be morphologically distinguished from the latter. Thus, in this study, we first established a molecular technique based on the polymerase chain reaction to accurately identify each species. In the subsequent field survey in 2015 with its application in the Sakasegawa River, Hyogo Prefecture, we found that *S. pedemontanum elatum* larvae hatch in stagnant water and subsequently advance into weakly flowing water. Our results indicated a change in the microhabitats during the larval developmental process, reflecting the need for a continuous spectrum of stagnant, transitional, and flowing water. Such aquatic environments with a spectrum of water conditions are disappearing in Satoyama, a rural farming area in Japan. This has endangered species such as *S. pedemontanum elatum* and *Oryzias latipes* (Beloniformes: Adrianichthyidae) by depriving them of their favorable habitats. For their conservation, it is necessary to develop methods to recover the traditional aquatic environments in Satoyama.

Key Words: Aquatic environment, Conservation, ITS, Molecular identification, Paddy fields, Satoyama, Specific primer

INTRODUCTION

Despite extensive research on the conservation of dragonflies and damselflies (e.g. Fox & Cham 1994; Samways 1994; New 1995; Samways & Steytler 1996; Corbet 1999; Butler & Maynadier 2008), some *Sympetrum* species (Odonata: Libellulidae) such as *S. evanescens*, *S. gracile*, *S. maculatum*, are still threatened (according to the reports by the International Union for Conservation of Nature [IUCN] 2015). *Sympetrum pedemontanum* (Müller in Allioni), which is distributed widely in the Eurasian continent and its neighboring islands, is also facing regional extinction or decrease in several European countries (IUCN 2015), although it is categorized as a Least Concern (LC) species in the IUCN Red List.

In Japan, its subspecies *S. pedemontanum elatum* (Selys) was one of the most common dragonflies in rural areas up to the 1970s (Aoki 1998, 2013) and was known as the most beautiful *Sympetrum* species in Japan. *S. pedemontanum elatum* and other dragonflies are generally symbols for courage, power, triumph, and happiness, and are often figures in Japanese traditional art, like Tanka (short Japanese poem) (Ishida et al. 1988; Corbet 1999). However, the

population has been rapidly decreasing, and this species is now listed in Japanese prefectural red lists as Extinct (EX) in Chiba Prefecture, Critical Endangered (CR) in Nagasaki Prefecture, Vulnerable (VU) in Tokyo Metropolis, and Near Threatened (NT) in many other prefectures (Association of Wildlife Research and EnVision 2013). To conserve them for sustenance of biodiversity as well as the cultural ecosystem services that they have provided for us in Japanese farmlands, it is necessary to understand the species-specific microhabitat use. Indeed, some studies along these lines have been reported for the Nagasaki (Kawachino 2015) and Hyogo (Yagi et al. 2006, Adachi et al. 2007) Prefectures, but few on larval microhabitat use.

Sympetrum pedemontanum elatum prefers open fields with weakly flowing streams in hilly areas (Maeto et al. 2003), such as the foot of Satoyama (Ishida et al. 1988; Aoki 2013), which is a rural farming landscape of Japan that traditionally provides habitats for many aquatic species (Katoh et al. 2009). Further, it have been known to be associated with either weakly flowing or shallow and stagnant water, especially in sunny, seepage-fed, terraced paddy fields, or slowly running ditches and channels (Ishida et al. 1988; Aoki 1998, 2013; Kondo et al. 2005; Yagi et al. 2006; Muraki 2010; IUCN 2015; Kawachino 2015).

Adults emerge in late June to early July (Ishida et al. 1988; Aoki 2013; Ozono et al. 2013), and are weak fliers that perch on grasses near the water until they mature in August (Ishida et al. 1988; Aoki 1998, 2013). The females form tandem pairs with males and lay eggs by dipping the tip of their abdomens in the edge of the water or in the nearby sediments (Ishida et al. 1988;

Aoki 2013; Ozono et al. 2013). They prefer to lay eggs in stagnant or slightly flowing rather than strongly flowing water (Aoki 2013; Higashikawa pers. observ.). The adults disappear by the end of November (Ishida et al. 1988; Yagi et al. 2006).

The larvae begin to appear in March (Yagi et al. 2006). Although these larvae cannot be morphologically discriminated from congeners, they are thought to be associated with flowing water, inhabiting pools along the edges of weak streams (Ishida et al. 1988; Kondo et al. 2005; Ozono et al. 2013). However, because oviposition behavior of this subspecies is often observed on either stagnant or very slowly flowing water that is connected with weakly flowing water (Aoki 2013; Higashikawa pers. observ.), we hypothesized that the developing larvae may move among these transitional, aquatic habitats. Indeed, terraced paddy fields, which are typically the main habitat of this species (Yagi et al. 2006; Kawachino 2015), have this precise spectrum of water conditions (Tabuchi et al. 1983; Kawachino 2015).

Larvae of *S. pedemontanum elatum* often coexist with an indistinguishable common congener *S. eroticum* (Selys). Therefore, it has not been possible to investigate the distribution and microhabitat use of larvae. In this study, we developed a molecular identification system using polymerase chain reaction (PCR amplification) (e.g., Hoogendoorn & Heimpel 2001; Itou et al. 2013) to discriminate between the larvae of *S. pedemontanum elatum* and *S. eroticum*. Once this discrimination was possible, we examined the seasonal microhabitat use of *S. pedemontanum elatum* larvae in a lowland stream in Japan.

MATERIALS AND METHODS

Study site

The larval distribution of *S. pedemontanum elatum* was studied at the Sakasegawa River, Takarazuka City, Hyogo Prefecture, Japan (Fig. 1). Although this river is not the species' main habitat (which is Satoyama, a rural farming area in Japan), its population in this river has been exceptionally abundant and stable in recent years (Yagi et al. 2006; Adachi et al. 2007). This small river flowing over the flat lowland area has heterogenous aquatic components, which are convenient for the investigation of microhabitat use by the larvae. We selected five study sites (1–5) along the river (Fig. 1), each having a set of stagnant, transitional, and strongly flowing zones within a distance of 3 m (Fig. 2). For each of the five sites, we set a sampling plot in each of the following four microhabitats: (A) shallow stagnant (water depth [WD], 3–7 cm; surface flow velocity [SFV], 0 cm/s; sediment [SED], dark silt); (B) very weakly flowing (WD, 5–8 cm; SFV, 4–7 cm/s; SED, mostly dark silt); (C) weakly flowing (WD, 7–11 cm; SFV, 7–15 cm/s; SED, white sand); and (D) deep and strongly flowing (WD, 20–50 cm; SFV, >30 cm/s; SED, sand and stones) (Fig. 2). (B) and (C) were separated by a submerged ridge.

Seasonal sampling and identification of larvae

Sampling of the larvae was conducted in the middle of every month from March to August 2015.

We collected sediments 1–3 cm deep from three 15 cm × 15 cm quadrats at least 0.5 m apart from each other within every sampling plot. To collect the sediment, a plastic net of 0.5-mm mesh size was vertically placed into the water, facing upstream. *Sympetrum* larvae were picked up from the collected sediment under a stereoscopic microscope (SZ2-ILST; Olympus, Japan).

We then measured the head width of the collected larvae under a digital microscope VHX-600 (Keyence, Japan). A middle leg was removed from each individual to provide DNA. Next, we identified the larvae by PCR, using the specific primer sets as described below.

Design of PCR primers specific to *S. pedemontanum elatum* and *S. eroticum*

Sampling for adults of the two species and extraction of nuclear DNA

In August 2014, we collected 30 *S. pedemontanum elatum* and *S. eroticum* adults each from the Hyogo Prefecture by nets. It is noteworthy that adult *S. pedemontanum* and *S. eroticum* are morphologically distinguishable. Alkaline elution procedure was then performed to extract nuclear DNA from the middle legs of these adults. Each middle leg was added to 20 µL of 50 mM NaOH in a PCR tube and incubated at 95 °C for 15 min. This was then neutralized with 20 µL of 200 mM Tris-HCl.

Polymerase chain reaction for the ITS1 region of DNA

We selected the nuclear ITS1 region for PCR amplification, since this region shows numerically more species specific variations than does the mitochondrial *COI* gene in *Sympetrum* (Futahashi 2011). The following primer set was used for the amplification of the ITS1 region of the two species: 5'-GGC CAA ACT TGA TCA TTT AG-3' (forward) and 5'-GCC GGC CCT CAG CCA G-3' (reverse) (Palumbi et al. 1991). PCR amplification was performed in a total volume of 25 μ L, containing 5 μ L of 2 mM dNTPs, 12.5 μ L of 2 $\times$ Buffer, 0.5 μ L of KOD FX Neo (Takara, Japan), 0.5 μ L of each 10 pM primer, 1.0 μ L of distilled water, and 5 μ L of the extracted template DNA. The mixture was first denatured at 94 $^{\circ}$ C for 2 min. The cycling conditions included 40 cycles of 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 68 $^{\circ}$ C for 1.5 min. The PCR products were then refined with the illustra GFX kit (GE Healthcare Life Sciences, UK).

DNA Sequencing

We sequenced the amplified DNA to examine the individual variations in the ITS1 region. Cycle sequences were done in a total volume of 20 μ L, containing 2 μ L of Big Dye Terminator (Applied Biosystems, U.S.A), 0.4 μ L of 10 pM primer (either forward or reverse), 4 μ L of 5 $\times$ sequencing buffer, 150 ng of the refined PCR product, and the required quantity of distilled water. The cycling conditions were as follows: initial denaturation at 96 $^{\circ}$ C for 2 min, followed

by 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. The DNA samples were then purified by ethanol precipitation, added to 20 µL of Hi Di Formamide, agitated for 15 s, and incubated at 95 °C for 2 min. The reaction mixtures were then quickly kept on ice before sequencing on the ABI3100 Genetic Analyzer (Applied Biosystems).

The wave data obtained were applied to the DNA Dynamo sequence analysis software (Blue Tractor Software, North Wales, UK) to modify the disorder of the waveforms in the contigs of forward and reverse reads. The sequences aligned by ATCG with ClustalW (clustalw.ddbj.nig.ac.jp/) were then submitted to the DNA Data Bank of Japan (DDBJ; Accession numbers LC107001–107018).

Primer design

Based on our sequence data and those for all 21 *Sympetrum* species in Japan (Futahashi 2011), we designed specific primer sets for *S. pedemontanum elatum* and *S. eroticum* using the web tool Primer-Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). All available sequences for all the Japanese *Sympetrum* species are shown in alignment in Fig. S1. The designed primer sets, HSP and HSE, for *S. pedemontanum elatum* and *S. eroticum*, respectively, are shown in Table 1. Two forward primers were designed for HSE (HSE - Fa/b) according to the variety of the specific sequences.

Species-specificity testing for the designed primers

We tested whether the HSP and HSE primer sets could accurately amplify the targeted ITS1 regions in the larvae of the two species. Towards this aim, we used three adult specimens for each species for the DNA samples. All the PCR reaction mixtures were similar to the 25- μ L solutions described above. For the HSP primer set, the DNA was denatured at 94 °C for 2 min; subsequent cycling conditions were 35 cycles of 94 °C for 15 s, 60.9 °C for 30 s, and 68 °C for 1 min. The cycling conditions for the HSE primer set were the same, except that the annealing temperature was 64.2 °C. We observed specific bands for each target amplicon in the appropriate sample on a 2% agarose gel.

We also confirmed that the primers correctly amplified the desired target regions by sequencing the above PCR products. Moreover, we confirmed the primer specificities by performing PCR for DNA samples from 12 Japanese *Sympetrum* species (*S. darwinianum*, *S. risi*, *S. infuscatum*, *S. frequens*, *S. depressiusculum*, *S. striolatum imitoides*, *S. parvulum*, *S. eroticum*, *S. pedemontanum elatum*, *S. speciosum*, *S. croceolum*, and *S. uniforme*) with these primer sets.

Statistical analysis

The distributions of *S. pedemontanum elatum* larvae in April, May, June and July were analyzed using the generalized linear mixed model (GLMM) in the IBM SPSS Statistics software

(version 22). Data for March and August were not included in the analysis because of the small sample sizes. The number of larvae in each plot as a target variable was modeled with a Poisson distribution and a log link. While the fixed variables were month, microhabitat (A–D), and the interactions between month and microhabitat, the site (1–5) was the random variable. The effect of each microhabitat in each month was also analyzed and pairwise mean comparisons were performed using sequential Bonferroni corrections.

The larval head widths were analyzed using the generalized linear model (GLM) in the same software (SPSS). Data for larvae from all plots in each microhabitat were pooled for each month. We used GLM to test the effects of microhabitats in each month, and the Tukey-Kramer multiple comparison was applied to compare means for the microhabitats. Further, the gradual changes in the head width from microhabitat (A) to (D) in each month were analyzed using Kendall's Tau rank correlation.

RESULTS

The specificity of HSP and HSE primers

The HSP and HSE primers produced amplicons of the desired molecular weights (Fig. 3). Sequencing of these amplicons confirmed the targeted ITS1 regions. Moreover, strict specificity of these primers were confirmed upon PCR amplification of DNA from 12 other

Japanese species of *Sympetrum* mentioned above (Fig. 4).

Seasonal changes in the distributions of larval *S. pedemontanum elatum* and *S. eroticum*

In all, 184 and 33 larvae of *S. pedemontanum elatum* and *S. eroticum*, respectively, were collected (Table S1). The numbers of *S. pedemontanum elatum* larvae in each plot is shown in Fig. 5. In March, the larvae were collected from only microhabitat (A). In April and May, larval abundance increased in (B) and (C). In June, most larvae were collected from (B) and (C), and only one larva each from (A) and (D). Similarly in July, most larvae were collected from (B) and (C), two from (D), and one from (A). No larvae were available in any of the microhabitats in August.

The effects of the microhabitat, month, as well as their interactions on the larval numbers were observed to be significant (Table 2). In particular, the effects of microhabitats on larval abundance in each month were significant except for July (Table 3), when the number of larvae was very small. The estimated mean number of larvae per plot in each microhabitat is shown in Table 4. In April, it was significantly more abundant in (A), (B), and (C) than in (D) (A vs D, $p = 0.006$; B vs D, $p = 0.008$; C vs D, $p = 0.004$ in pairwise mean comparisons between microhabitats). However, larvae were significantly more abundant in (A) and (B) than in (C) and (D) in May (A vs C, $p = 0.032$; A vs D, $p = 0.013$; B vs C, $p = 0.033$; B vs D,

$p = 0.013$). In June and July, no significant difference in abundance was observed among the microhabitats.

Season–and microhabitat–dependent variations in larval body size of *S. pedemontanum elatum*

The mean head width of *S. pedemontanum elatum* larvae in each microhabitat in each month except for March is shown in Fig. 6 (raw data are shown in Table S2). In May, the head widths significantly differed among the microhabitats ($F_{2,60} = 11.490$; $p < 0.001$), and were larger in (B) and (C) than in (A). In April, June and July, the head widths did not differ significantly between the microhabitats ($F_{2,93} = 1.232$; $p = 0.297$ in April; $F_{3,21} = 2.176$, $p = 0.128$ in June; $F_{3,9} = 0.798$, $p = 0.525$ in July), but a gradual increase of means from (A) to (D) was observed in each of these months. There was a significant positive correlation between the order of microhabitat (A) to (D) and the head width in April and May (Tau = 0.311, $n = 83$, $p < 0.001$ in April; Tau = 0.383, $n = 61$, $p < 0.001$ in May). In June and July, there was no significant correlation between them (Tau = 0.212, $n = 21$, $p = 0.240$ in June; Tau = 0.267, $n = 13$, $p = 0.255$ in July).

DISCUSSION

Populations of *S. pedemontanum elatum* in Japan have been rapidly decreasing since the

1970s, especially in Chiba Prefecture (EX), Nagasaki Prefecture (CR), Tokyo Metropolis (VU), and many other prefectures (NT), as reported in Japanese prefectural red lists (Aoki 1998, 2013; Association of Wildlife Research and EnVision 2013). In order to help develop strategies to conserve this species, we attempted to understand their ecology by studying their larval seasonal microhabitat use in the Sakasegawa River, Takarazuka City, Hyogo, Japan. First, referring to some studies mentioning the efficacy of insect species identification by PCR (Hoogendoorn & Heimpel 2001; Itou et al. 2013), we successfully designed species-specific primers for PCR to accurately distinguish larval *S. pedemontanum elatum* from its congener *S. eroticum*.

The results of field surveys and the genetic identifications allow us to hypothesize the following seasonal distribution of larvae. In March, almost all newly hatched *S. pedemontanum elatum* larvae are in essentially stagnant water that have a connection to weakly flowing water. In April and May, most of larger larvae move into weakly flowing water, but some slowly growing individuals remain in the lentic zone. In June and July, most larvae live in weakly flowing water. We found significantly more larvae in stagnant water than in flowing water in May, but by June and July, the trend was reversed. We found more larvae in deeper, moving water; however, the stagnant pools never reached this depth. Larvae might change their microhabitat in response to water movement, rather than depth, although other factors, such as water temperature and prey availability, may also play a role.

Interestingly, only a few larvae were collected in strongly flowing water in any given month, which indicates that *S. pedemontanum elatum* rarely use such zone during its growth period.

Previous studies have shown that although *S. pedemontanum elatum* larvae mainly live in flowing streams, they are often observed in stagnant water as well, perhaps because adults prefer to lay eggs into stagnant water (Ishida et al. 1988; Aoki 1998; Kondo et al. 2005; Muraki 2010). Our results address this discrepancy by explaining that the larvae probably changed their microhabitat during development, thus presenting themselves in a continuous spectrum of stagnant, transitional, and flowing water. Several studies have examined the movement of Odonata larvae within ponds (Lawton 1970; Pierce 1988; Duffy 1994; Corbet 1999; Johansson & Suhling 2004) or streams (Corbet 1999; Lancaster & Downes 2103), but no information was available on the larval movement of *Sympetrum* species from stagnant to flowing water during development.

The morphology of *S. pedemontanum elatum* larvae closely resembles that of other *Sympetrum* species, which are primarily lentic. Accordingly, its body shape should be suited for development in stagnant water. Larvae may face energetic costs when moving from stagnant to flowing water, but the different microhabitat might offer benefits like better prey availability, temporary refuge from predators, and increased water oxygenation. In the Sakasegawa River, prey organisms, such as juvenile fish like *Rhinogobius flumineus* or some shrimp species, are larger in flowing water and are higher quality than prey like the small

isopod *Asellus hilgendorfi* in stagnation (Higashikawa pers. observ.). Predators are abundant in both of stagnation and flowing water, and include the large dragonfly larvae of *Anotogaster sieboldii* (stagnant) and predatory fish like *Odontobutis obscura* (flowing) (Higashikawa pers. observ.). However, *S. pedemontanum elatum* larvae take refuge in spaces among stones or stalks of plants, which are more abundant in flowing water. Flowing water also has higher levels of oxygen, and larvae might move out of stagnant water as their oxygen demands increase. Mayfly nymphs (Ephemeroptera) are known to move towards strongly flowing water as their need for oxygen increases (Wiley & Kohler 1980). Similar seasonal microhabitat use by larvae may also be observed in some other *Sympetrum* species that are said to live in both stagnant and flowing water, such as *S. haritonovi* (Schröter 2010), *S. madidum*, *S. nigrifemur*, *S. nigrocreatum*, and *S. sinaiticum* (IUCN 2015).

In Japan, *S. pedemontanum elatum* prefers terraced paddy fields in hilly areas (Kondo et al. 2005; Yagi et al. 2006; Kawachino 2015), which have a characteristic water system called “plot-to-plot irrigation” (Katano et al. 2001; Ishii & Sakuma 2008). In this system, water flows in from the upper steps and flows out into the lower steps of the fields. Each paddy field is used as a part of the “irrigation ditch”, and thus, the paddy fields can simultaneously provide both shallow stagnant and weakly flowing water connected by a transitional zone. Similarly, in traditional paddy fields in the flatlands of Japan, where *S. pedemontanum elatum* was once abundant (Ishida et al. 1988; Aoki 2013), the water enters into and is drained from

the paddies via shallow earth or wooden ditches. The water level in these ditches is similar to that in paddies (Fujioka & Lane 1997). This ensures a continuous water spectrum of stagnant, transitional, and weakly flowing water within and among paddy fields (Tabuchi et al. 1983). In the Sakasegawa River, the water conditions are similar to those in terraced or traditional paddy fields, a critical factor that enables *S. pedemontanum elatum* larvae to flourish.

Oryzias latipes (Belontiiformes: Adrianichthyidae), a Vulnerable (VU) fish species in Japan (Association of Wildlife Research and EnVision 2013), was one of the most common fish dwelling in and around paddies until the 1970s (Hata 1998; Kondo et al. 2005; Fukuda 2009). It could move among paddy fields through the irrigation ditches (Hata 1998, 1999), using water flowing very weakly at 4 cm/s or less (Fukuda 2009). Therefore, it follows that *O. latipes* had a microhabitat requirement similar to that of *S. pedemontanum elatum* larvae in this study. Thus, we conclude that such aquatic environments in and around traditional paddy fields, containing both stagnant and weakly flowing water, are probably essential to some endangered species in Satoyama, such as *Gynacantha japonica* (Odonata), *Pelophylax nigromaculatus* (Anura), *Hynobius nebulosus*, and *H. dunni* (Urodela), Japan (Fujioka & Lane 1997; Tanaka 1999; Mukai et al. 2005; Katoh et al. 2009).

Unfortunately, terraced paddy fields in Japan have been decreasing since the 1960s (Yoshikawa 2006) because of their low productivity and requirement of intensive labor for maintenance (Nakajima 1996; Ishii & Sakuma 2008). Further, the traditional water systems of

paddy fields in the flatlands have also been changed to contemporary water systems post the 1960s (Fujioka & Lane 1997). In these contemporary systems, the water in the paddy fields is usually stagnant, especially during non-irrigation periods, owing to the functional difference in irrigation and drainage ditches (Fujioka & Lane 1997; Katano et al. 2001). Perhaps, due to such a result of changes in the aquatic environment in Satoyama, *S. pedemontanum elatum*, *O. latipes*, and many other aquatic species have lost their habitats and are in the process of depletion since the 1970s.

While habitat loss has caused the countrywide decrease of *S. pedemontanum elatum* in Japan, several rivers, including the Sakasagawa, still provides an appropriate combination of stagnant and weakly flowing water, which favors the growth of the larvae. These rivers have played an important role in temporarily maintaining populations of the dragonfly, and perhaps other aquatic species as well, under threat of regional extinction after changes in Satoyama water system. However, such rivers are scarce in Japan because most Japanese rivers are too steep to provide a wide spectrum of water conditions within a compact area.

Therefore, to prevent further decrease in the population of these endangered species, it is not only necessary to maintain the existing terraced paddy habitats (e.g., Kawachino 2015), but also crucial to develop and apply methods to recover the aquatic environments that were once characteristic of the farming landscape of Satoyama.

A number of methods have been used to make artificial habitats for threatened fish and

arthropods in Satoyama, and these should be applicable to conservation of *S. pedemontanum elatum* and other cohabiting species: i) Installing waterways within and between modern paddy fields or ditches (e.g., Hata 1999); ii) Constructing artificial stagnations along rivers and controlling flow regulation with dikes or levees to conserve fish living in weakly flowing water, such as *Biwia zezera* and *Opsariichthys platypus* (Ikeuchi & Kanao 2003).

In addition, public support for conservation is very important. Shimanto City in Kochi Prefecture has a dragonfly sanctuary called Dragonfly Kingdom, a well-known nature park popular with tourists (Sugimura 1996). The park includes a mosaic of different vegetation types and aquatic environments to maximize habitats for dragonfly and damselfly species. This “Shifting Mosaic System” of habitats facilitates the conservation of water and wetland wildlife (Asami et al. 2001). Conservation activities, whether in rural or urban areas, must proceed with support and cooperation from farmers and regional public agents and be based on the scientific recognition of decreasing aquatic biodiversity in Satoyama.

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FIGURES AND TABLES

(a)



(b)

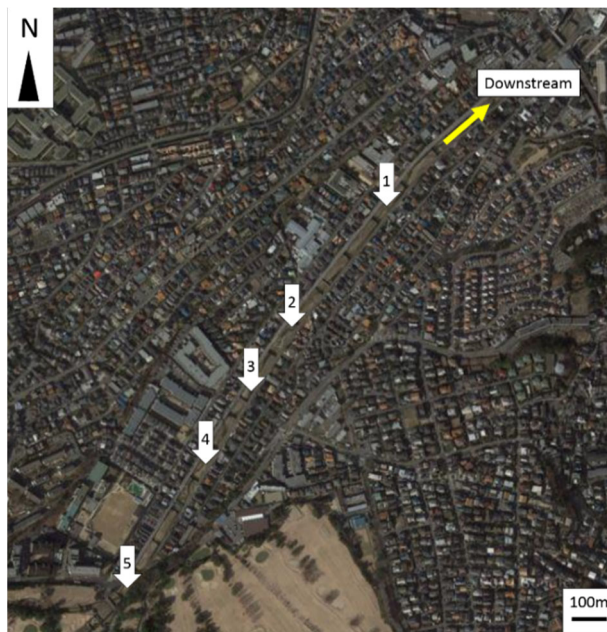
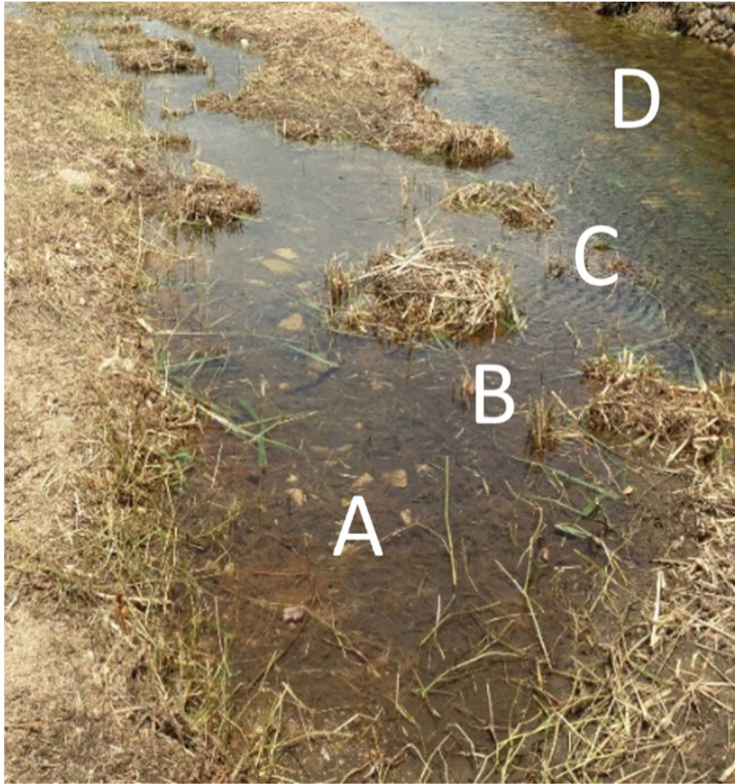


Fig. 1. (a) Ground-level view of the Sakasegawa River. (b) Location of five study sites (indicated with arrows) at Takarazuka City, Hyogo Prefecture, Japan. Latitude/longitude, $34^{\circ}47'45''/135^{\circ}20'50''$; altitude, 60–90m above sea level; average slope, 3.7%. Map data, ©2015 Google Earth.

(a)



(b)

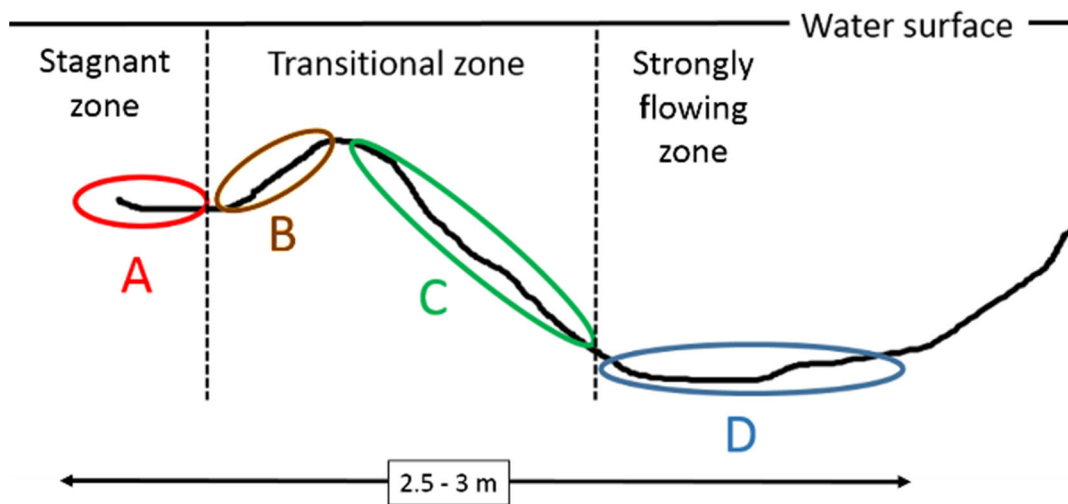
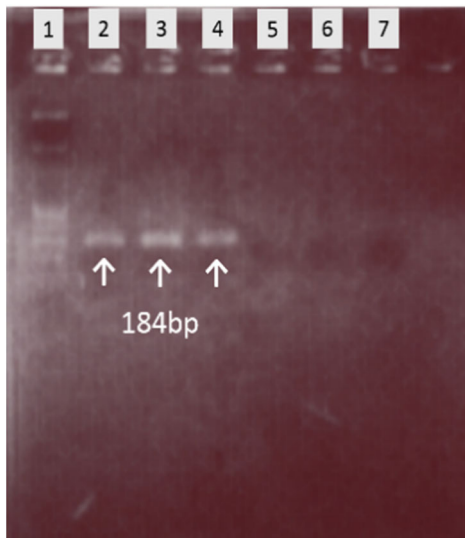


Fig. 2. (a) Representative picture of study site 4, showing the stagnant (A), transitional (B and C), and strongly flowing (D) zones. (b) Schematic cross-sectional view of a study site consisting of the four microhabitats (A–D). The solid curve represents the riverbed.

(a)



(b)

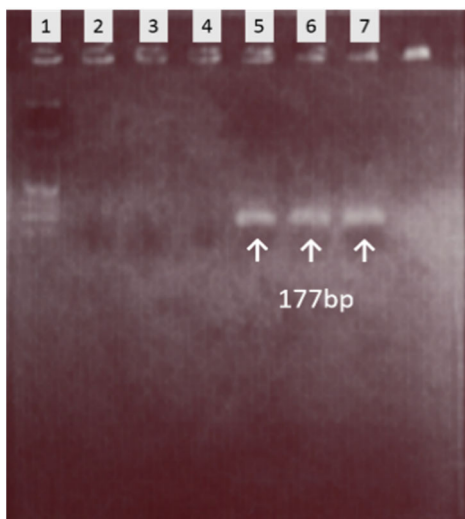
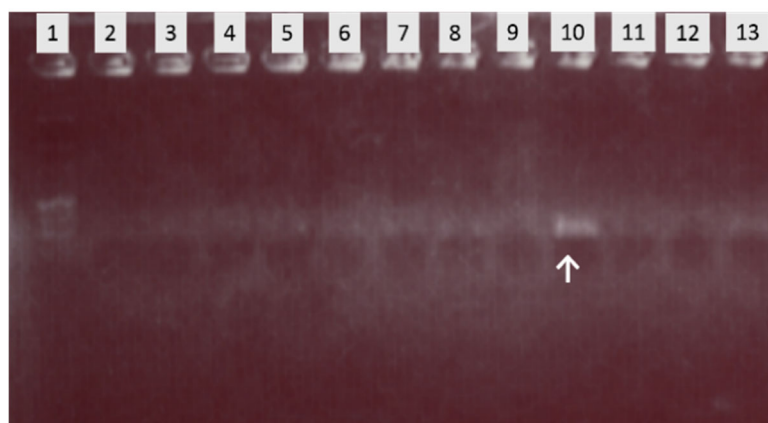


Fig. 3. Representative images of agarose gels showing amplifications products for ITS1 obtained by polymerase chain reaction (PCR) with primer sets HSP (a) and HSE (b) specific to *Sympetrum pedemontanum elatum* and *S. eroticum*, respectively. Lane 1, 100-bp molecular size marker; lanes 2 - 4: PCR products for *S. pedemontanum elatum*; lanes 5 - 7: PCR products for *S. eroticum*. The lack of bands for the two species in the respective gels indicates the specificities of the primer sets.

(a)



(b)

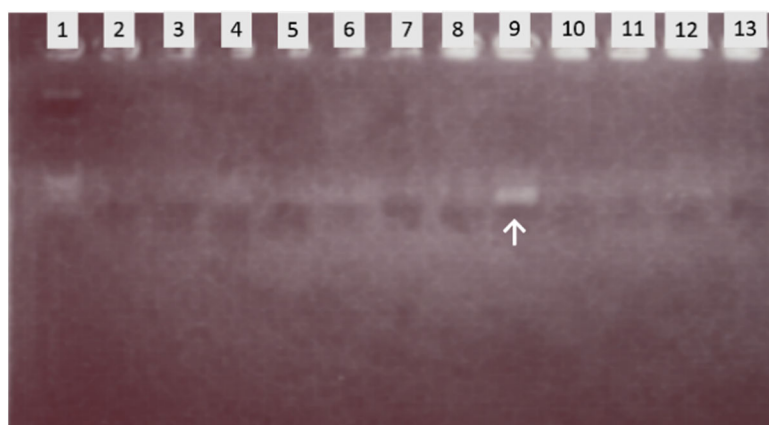


Fig. 4. Representative images of agarose gels illustrating the species-specificity of primer sets HSP (a) and HSE (b) specific to *Sympetrum pedemontanum elatum* and *S. eroticum*, respectively. Lane 1 shows the 100-bp molecular size marker. Polymerase chain reaction was performed, and the results are shown for the following species: lane 2, *S. darwinianum*; lane 3, *S. risi*; lane 4, *S. infuscatum*; lane 5, *S. frequens*; lane 6, *S. depressiusculum*; lane 7, *S. striolatum imitoides*; lane 8, *S. parvulum*; lane 9, *S. eroticum*; lane 10, *S. pedemontanum elatum*; lane 11, *S. speciosum*; lane 12, *S. croceolum*; and lane 13, *S. uniforme*.

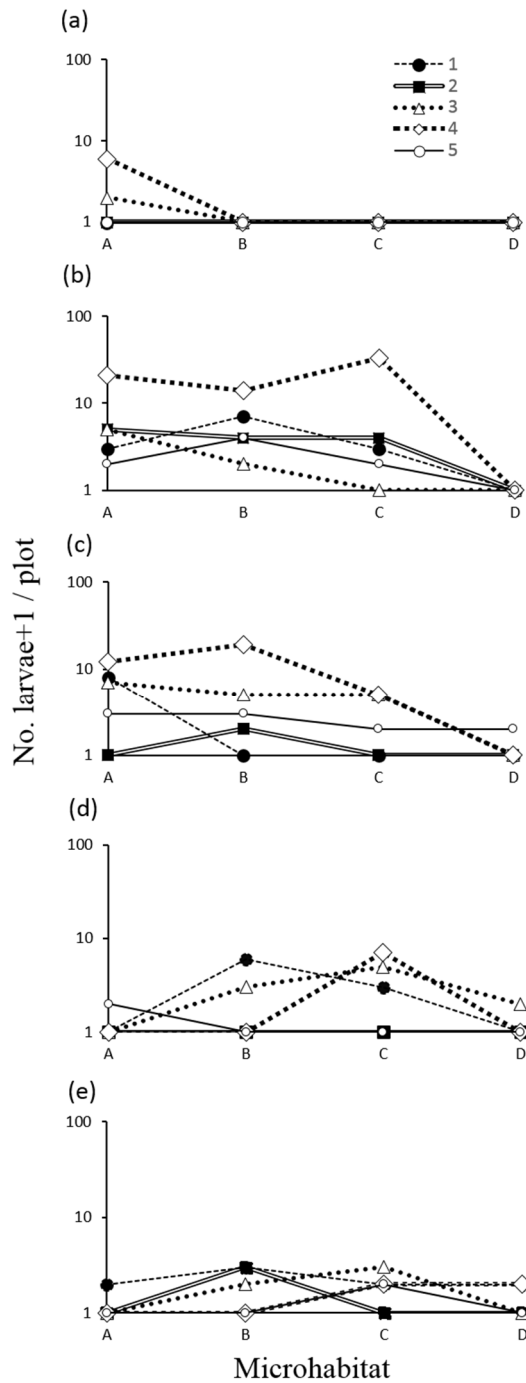


Fig. 5. Number of *Sympetrum pedemontanum elatum* larvae in each plot (three quadrats 15 cm $\times$ 15 cm each) at the five study sites in March (a), April (b), May (c), June (d), and July (e) 2015. 1–5 in the key to symbols indicate the sites as shown in Fig. 2.

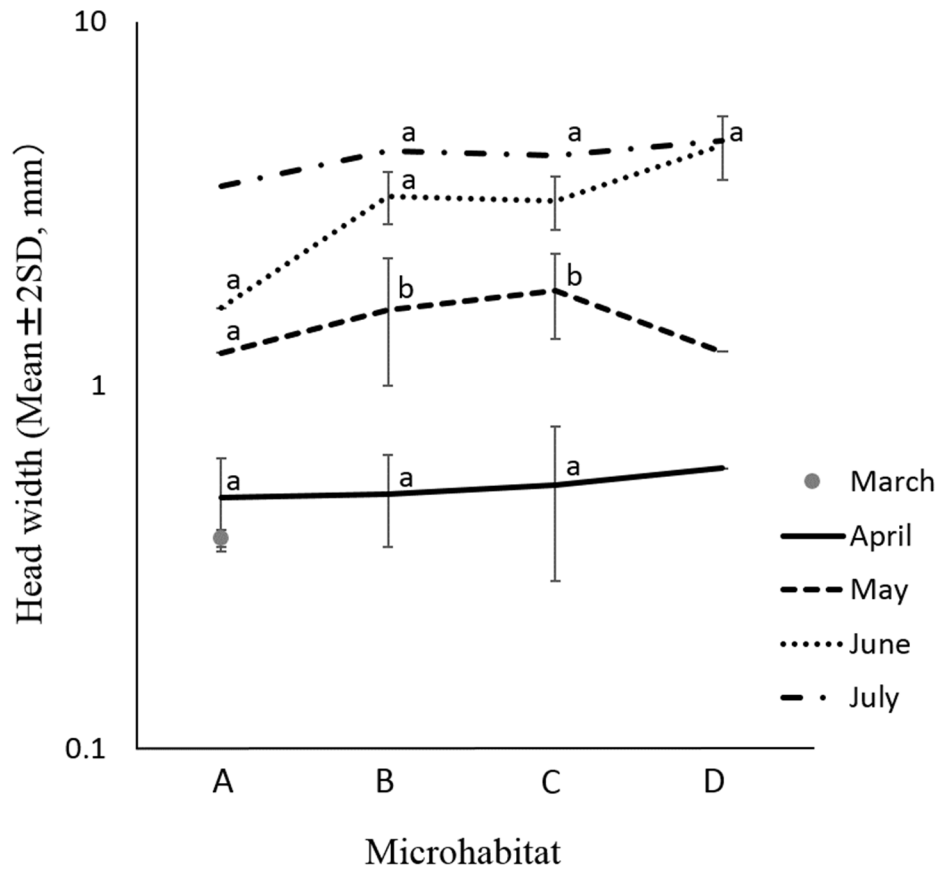


Fig. 6. Mean head width (mm) of *Sympetrum pedemontanum elatum* larvae in each microhabitat (A–D) from April to July. The vertical line indicates ± 2 SD. Means with the same letter are not significantly different within the same month (Tukey Kramer multiple comparison, $p > 0.05$)

Table 1. Species-specific primers designed for amplification of a partial ITS1 region from the nuclear DNA of *Sympetrum pedemontanum elatum* and *S. eroticum* by polymerase chain reaction (PCR)

Species	Primer	Sequence (5'→3')	PCR product length (bp)	Optimal annealing temperature for the primer set (°C)
<i>S. pedemontanum elatum</i>	HSP-F	CGTATGGAGAGAAAAACACA	184	60.9
	HSP-R	CTCTCTCGAGAATCCTCAAT		
	HSE-Fa	TCATTAACGAACGCTCCC		
<i>S. eroticum</i>	HSE-Fb	TCATTAACGAACGCTCTCC	177	64.2
	HSE-R	ACCGACCGGGATAAGTT		

Table 2. Results of generalized linear mixed model analysis of the effects of fixed variables on the larval abundance of *Sympetrum pedemontanum elatum*

Fixed variable	F ratio	df1	df2	Probability
Microhabitat	8.940	3	59	<0.001
Month	6.205	3	59	0.001
Microhabitat [×] Month	2.656	9	59	0.012

Table 3. Results of generalized linear mixed model analysis of the effect of microhabitats on the larval abundance of *Sympetrum pedemontanum elatum* in each month

Month	F _{3, 59}	Probability
April	4.436	0.007**
May	5.889	0.001**
June	3.581	0.019*
July	1.119	0.349

Table 4. Estimated means (95% confidence interval [CI]) of larval abundance of *Sympetrum pedemontanum elatum* per plot in each microhabitat (A–D) from April to July 2015

Month	Microhabitat							
	A		B		C		D	
	Estimated mean number	95%CI	Estimated mean number	95%CI	Estimated mean number	95%CI	Estimated mean number	95%CI
April	3.30 ^a	0.98 - 11.10	4.99 ^a	1.40 - 17.69	4.04 ^a	1.21 - 13.45	0.11 ^b	0.01 - 1.20
May	3.59 ^a	1.18 - 10.96	3.46 ^a	11.30 - 10.57	1.24 ^b	0.36 - 4.35	0.14 ^b	0.01 - 1.46
June	0.47 ^a	0.10 - 2.30	2.23 ^a	1.08 - 4.63	3.19 ^a	1.73 - 5.88	0.47 ^a	0.01 - 2.30
July	0.45 ^a	0.11 - 1.85	1.14 ^a	0.47 - 2.77	1.04 ^a	0.41 - 2.63	0.55 ^a	0.15 - 1.97

Means with the same letter are not significantly different within the same month (sequential Bonferroni corrections, $p > 0.05$)