1 **Title:**

2 **OdoCocktail-Japan: Primer sets to enrich environmental DNA of Japanese Odonata species**

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20 **Abstract**

21 Monitoring is essential to conserve and recover biodiversity. Environmental DNA (eDNA)-based
22 metabarcoding analysis has been recognized as a cost-effective and efficient method to monitor
23 species, but it still has limitations, such as insufficient reference databases and lack of primer sets
24 suitable to detect target species. As biological indicators of freshwater habitats, Odonata species have
25 been a monitoring target. As of yet, however, no metabarcoding primer sets can efficiently detect
26 them. In the present study, we developed three primer sets (named OdoCocktail-Japan) to amplify a
27 partial 16S rRNA sequence of Japanese Odonata species and assessed the performance of the primer
28 sets using eDNA samples collected in the field. Each primer set (versions 1, 2, and 3) was designed to
29 cover 203 of the 204 Japanese Odonata species, whose 16S rRNA sequences are available in a public
30 database. The forward and reverse primers of versions 1 and 2 were designed to match four and five
31 regions, respectively. On the other hand, those of version 3 were designed to match a single pair of
32 regions, and the 3′-end base of forward primers was used to identify Odonata species. In silico PCR
33 suggested that OdoCocktail-Japan primer sets cover 60–75% (including 203 Japanese Odonata) of all
34 Odonata sequences available in the public database. The assessment using field samples showed that
35 OdoCocktail-Japan improved the efficiency of Odonata detection in eDNA metabarcoding: the
36 proportion of Odonata reads in high-throughput sequencing reached 32.7–85.6%, with OdoCocktail
37 version 1 enriching Odonata sequences most efficiently among all versions. In addition, detected
38 species were consistent with those encountered during water sampling or historical records for each
39 sampling site. However, when Odonata eDNA was relatively rare or absent in samples, all versions
40 amplified Ephemeroptera and Plecoptera as alternative templates in PCR. Thus, OdoCocktail-Japan
41 primer sets offer high performance with samples from lentic environments, although further
42 improvements are needed for sampling lotic environments, where Ephemeroptera and Plecoptera are
43 usually abundant. We discuss how OdoCocktail-Japan can be modified to detect Odonata species
44 from countries and areas outside Japan.

45 **Keywords:** eDNA metabarcoding, aquatic insect, 16S rRNA, species composition

Introduction

More than 10% of all known species inhabit freshwater environments, yet one-quarter of these species are threatened with extinction due to pollution, dams, water extraction, agriculture, and invasive species (Sayer et al. 2025). To halt and reverse the loss of freshwater biodiversity, conservation and restoration of freshwater habitats as well as the monitoring of biodiversity to assess recovery are necessary (Arthington 2021). Although a variety of sectors, such as citizens, companies, and administrations, conduct conservation actions, the monitoring process requires the involvement of experts. Molecular identification based on environmental DNA (eDNA) is a promising method to solve the bottleneck and makes biodiversity monitoring accessible to many sectors (Pascher, Švara, and Jungmeier 2022; Clarke et al. 2023).

Theoretically, eDNA-based analysis can detect all organisms that shed their DNA into the environment. To take advantage of this benefit, universal primers that match a broad range of species have been developed, such as universal primer sets for fish (e.g., Miya et al. 2015; Valentini et al. 2016), birds (Ushio et al. 2018; Liang et al. 2026), and insects (Leese et al. 2021; Takenaka et al. 2023). For narrower groups, however, universal primers would be inefficient because they can produce many non-target sequences through high-throughput sequencing. For example, when target organisms are relatively rare in the environment, thus shedding relatively little DNA, only a few sequences of the target species can be obtained. In such cases, researchers require primers specific to the target species group. While primer sets that amplify the DNA of relatively small species groups, such as eels and whales, have been developed (Takeuchi et al. 2019; Ushio et al. 2025), efficient primer sets have not yet been developed for many taxonomic groups that serve as ecological indicators.

Odonata species, dragonflies and damselflies, are indicator taxa and targets for biodiversity monitoring (Lee Foote and Rice Hornung 2005; Miguel et al. 2017; Šigutová, Šipoš, and Dolný 2019). Odonata faunal diversity and abundance often reflect the quality of environments (Miguel et al.

72 2017; Baba, Kusumoto, and Tanaka 2019; Rocha-Ortega, Rodríguez, and Córdoba-Aguilar 2019), and
73 there is a growing need to monitor Odonata fauna to assess the quality of freshwater habitats (Fan,
74 Qin, and Hu 2025). In addition, Odonata are culturally important in Japan, with these attractive insects
75 captured in mythologies, samurai helmets and nursery rhymes (Katayama and Baba 2020; Hoshina
76 2022; Su 2022) and revered as adept hunters, particularly of agricultural pests in rice paddies and
77 disease vectors such as mosquitoes (Priyadarshana and Slade, 2023)—making their conservation even
78 more salient. However, Odonata nymphs are sometimes difficult to identify to species due to their
79 morphological similarity, and some species are especially rare. Therefore, primer sets for eDNA
80 metabarcoding of Odonata species could be useful. In addition, mitochondrial 16S rRNA sequences
81 are available for almost all Japanese Odonata species (i.e., 203 of the 204 species), meaning that an
82 almost complete reference database is available for Japanese Odonata species if metabarcoding
83 primers were to target the 16S rRNA region.
84 In this study, we developed primer sets for enriching amplicons of Odonata sequences. Although
85 insect universal primer sets have been used to assess Odonata diversity in eDNA studies, the primer
86 sets' efficiency is low when Odonata species are less abundant in the environment and the primers
87 may not cover all the species targeted. Thus, we developed primer sets that match all Odonata species
88 occurring in Japan and assessed their efficiency to detect Odonata species using samples collected
89 from paddy fields, biotopes, ponds, and an upstream river. Because of the completeness of reference
90 data for the gene, we explored sequences of the mitochondrial 16S rRNA gene of Japanese Odonata
91 species to specify the regions suitable for enriching Odonata amplicons. In addition, we discuss how
92 the proposed primer sets can be extended to detect Odonata species even in areas outside of Japan.
93

94 **Materials and Methods**

95 **Primer design and in silico PCR**

96 To develop a primer set, we searched the NCBI nucleotide database using the search terms
97 "Odonata[Organism] AND 16S" and downloaded all retrieved sequences. Because our main purpose

98 was to develop primer sets covering all Japanese Odonata species, we first created a dataset including
99 only Japanese Odonata species and examined the available regions of the 16S rRNA sequence for all
100 Japanese species. We then searched the entire downloaded Odonata dataset for sequences that
101 contained these available regions, and the matching sequences were added to the Japanese Odonata
102 dataset. Multiple sequence alignment was conducted using MAFFT (Katoh and Standley 2013) with
103 the default parameter settings in UniPro UGENE (Okonechnikov et al. 2012). In addition, we
104 downloaded 16S rRNA sequences of Coleoptera, Diptera, Ephemeroptera, Plecoptera, Hemiptera,
105 Crustacea, and segmented worms from the NCBI nucleotide database to assess primer specificity
106 against non-target taxa.

107 The procedure used to identify sequences suitable for primer design followed a heuristic
108 approach. In general, we searched Odonata sequences for candidate primer regions with appropriate
109 properties (e.g., approximately 50% GC content), evaluated their Tm values using Primer3
110 (Untergasser et al. 2012), and then assessed the potential amplification of non-Odonata sequences by
111 performing in silico PCR using obipcr (Boyer et al. 2016). For the in silico PCR, up to four
112 mismatches between primers and template sequences were allowed, except for the last three bases at
113 the 3′ ends of both the forward and reverse primers. In addition, we retrieved amplicons between 50
114 and 500 bp in length.

115 Although we identified regions that were highly conserved among Japanese Odonata species in
116 the preliminary screening, these regions also annealed sequences from non-Odonata taxa. Therefore,
117 our strategy was to identify multiple regions, each of which matched relatively small subsets of
118 Japanese Odonata species (e.g., closely related species or genera). Multiplexing such primers was
119 expected to provide coverage of all Japanese Odonata species. Based on this strategy, we developed
120 three primer sets: versions 1, 2, and 3 (hereafter “v1,” “v2,” and “v3,” respectively).

121 For v1, we selected primer regions in which the 3′ ends were relatively variable not only among
122 insect orders but also within Japanese Odonata species, whereas the 5′ ends were relatively conserved
123 across insect orders. The v2 set was a modified version of v1: the v2 primers were divided into

124 combination 1 and combination 2, and each combination was used separately in PCR to reduce
125 amplification of non-target species. When designing v3, we aligned sequences from six insect orders
126 (Odonata, Ephemeroptera, Plecoptera, Hemiptera, Diptera, and Coleoptera) and searched for
127 Odonata-specific regions. Primers were then designed at conserved regions for the forward and
128 reverse primers. In addition to these primers, we searched Odonata sequences for regions conserved
129 among Odonata species that could serve as inner primers to reduce amplification of non-target
130 organisms in a nested PCR. Such a nested PCR can also generate amplicons of suitable length for
131 high-throughput sequencing on the Illumina platform.
132
133 **Field eDNA sampling, eDNA extraction, and library preparation**
134 We assessed whether the primer sets increase the proportion of Odonata-derived amplicons using
135 field-collected samples. eDNA samples were collected from paddy fields, ponds, containers (small
136 artificial biotopes) for Odonata conservation, and upstream sites of a river (Table 1; Fig. 1). The
137 paddy field samples were originally collected for another study, and some of them (Paddies 8–10)
138 were used in a previous study (Yamamoto et al. 2025). Field sampling and eDNA extraction followed
139 Minamoto et al. (2021). Briefly, up to 500 mL of water was filtered using a Sterivex filter (pore size
140 0.45 µm; Merck Millipore, Burlington, MA, USA). To preserve the genetic material on the filters, 1.6
141 mL of RNAlater (Invitrogen, Waltham, MA, USA) was added to each Sterivex unit. Samples were
142 kept in a cool box with ice packs during fieldwork and stored at –20 °C in the laboratory. eDNA was
143 extracted from the Sterivex filters using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany)
144 following the protocol of Minamoto et al. (2021).
145 We prepared the amplicon libraries using a three-step PCR workflow: a pre-amplification PCR, a
146 first PCR with inner primers, and an indexing PCR (second PCR) (Fig. 2). The primer sets designed
147 in this study were used in the pre-amplification PCR, and the resulting products were subjected to the
148 first PCR. In the second PCR, 8-bp index sequences and Illumina adapter sequences were added to the

149 amplicons. In the following, we describe the conditions for the pre-amplification, first, and second
150 PCRs.
151 Pre-amplification PCR was performed in triplicate for each sample in a 15-µL reaction containing
152 1× KOD One PCR Master Mix (TOYOBO, Osaka, Japan), 0.3 µM of each primer, and 2 µL of eDNA
153 solution. The thermal cycling conditions consisted of 20 cycles of 98 °C for 10 sec, annealing at 58 °C
154 for v1 and v2 and 54 °C for v3 for 5 sec, and 68 °C for 5 sec (Table 2).
155 The first PCR was conducted in a 12-µL reaction containing 1× KOD One PCR Master Mix, 0.3
156 µM of each primer, and 2 µL of the pre-amplified sample. In the present study, the three replicates
157 from the pre-amplification PCR were used separately in the first PCR. The thermal cycling conditions
158 consisted of 25 cycles of 98 °C for 10 sec, 58 °C for 5 sec, and 68 °C for 5 sec. After the first PCR,
159 the three replicates of each sample were pooled. The pooled products were purified using
160 MagExtractor PCR & Gel Clean Up (TOYOBO) for v1 and by double-sided size selection using
161 NucleoMag NGS Clean-up and Size Select (Macherey-Nagel, Düren, Germany) for v2 and v3 (ratio
162 of PCR solution:NucleoMag = 1:0.6 for right-side selection and 1:0.95 for left-side selection). The
163 purified samples were then diluted 10-fold with pure water.
164 The second PCR was conducted in a 30-µL reaction containing 1× KAPA HiFi HotStart
165 ReadyMix (KAPA Biosystems, Wilmington, MA, USA), 0.3 µM of each index primer, and 6 µL of
166 the diluted sample. The thermal cycling conditions for the second PCR were as follows: 95 °C for 3
167 min; 15 cycles of 98 °C for 20 sec and 72 °C for 15 sec; and a final extension at 72 °C for 5 min. The
168 second-PCR products were purified by single-sided size selection using NucleoMag NGS Clean-up
169 and Size Select (PCR solution:NucleoMag = 1:0.9), and equal molar amounts of the purified products
170 were pooled. The resulting amplicon libraries were sequenced on an iSeq 100 sequencer (Illumina,
171 San Diego, CA, USA).
172 To assess cross-contamination, we included negative controls in each PCR batch for the v1 and v3
173 primer sets. We did not include a negative control in one PCR batch using the v2 primer set due to an
174 operational error; however, we did include seven extraction blanks in that batch. Therefore, we used

175 these extraction blanks to assess cross-contamination during the operation for PCR. Because the aim
176 of this study was to evaluate the performance of the primer sets rather than to examine ecological
177 characteristics of Odonata communities, field blanks were not subjected to PCR.
178
179 **Field observations of Odonata species at sampling sites**
180 To verify the validity of the results obtained through eDNA analysis, we photographed and recorded
181 all Odonata species observed during water sampling. In addition, we collected exuviae found during
182 sampling and brought them back to the laboratory for identification. The containers surveyed at
183 Okegayanuma (Table 1) were installed for the conservation of the dragonfly *Libellula angelina*,
184 categorized as Threatened IA by the Japanese Ministry of the Environment (equivalent to Critically
185 Endangered [CR] on the IUCN Red List). The species composition of Odonata nymphs within the
186 containers is regularly surveyed by a conservation organization, and we were provided with this
187 information.
188
189 **Data analysis**
190 The Illumina adapter sequences and polyG tails were removed from the obtained sequencing reads
191 using Fastp (Chen et al. 2018; see also Supplementary materials for detailed scripts). We discarded
192 sequencing reads lacking primer sequences, along with their paired reads, using Seqkit (Shen et al.
193 2016). The retained reads were processed using the R package DADA2 (Callahan et al. 2016)
194 implemented in R version 4.4.1 (R Core Team 2024). In the DADA2 workflow, we further filtered the
195 reads using the *filterAndTrim* function with the options maxN = 0, maxEE = c(2,2), and truncQ = 2.
196 Machine-learning-based error correction was performed using the *learnErrors* and *dada* functions,
197 and R1 and R2 reads were merged using the *mergePairs* function. Chimeric sequences were then
198 removed using the *removeBimeraDenovo* function. Finally, primer sequences were removed from the
199 amplicon sequence variants (ASVs) obtained by the DADA2 analysis.

200 We searched the NCBI core nt database for the ASVs using the web version of BLASTn
201 (Camacho et al. 2009; accessed February 2026). When a BLASTn result for a queried ASV showed
202 mismatch = 0, percent identity ≥ 98%, and alignment length ≥ 130 bp, we assigned the hit to the ASV.
203 If the BLASTn results did not include a hit with mismatch = 0, we allowed at most mismatch = 1.
204 When a BLASTn result suggested multiple species (e.g., due to identical 16S rRNA sequences among
205 closely related species), the ASV was assigned to all of the corresponding species. ASVs assigned to
206 the same taxon were then merged into an operational taxonomic unit (OTU). Finally, we obtained a
207 data matrix (i.e., OTUs × read counts). These analyses were conducted using custom scripts (see
208 supplementary materials) and Taxonkit (Shen and Ren 2021). One of custom scripts
209 (blast_xml2_to_csv.R) was developed to convert BLAST results from XML2 format into CSV tables,
210 with assistance from generative AI.

211 **Results**

212 **Primers and in silico PCR**

213 We specified three sets of pre-amplification primers (v1, v2, and v3) and one pair of first-PCR
214 primers (inner primers; see Table 3). The v1 set consisted of 7 forward primers and 19 reverse
215 primers. The maximum difference in their Tm values was 5.6 °C. Both the forward and reverse
216 primers were selected from two distinct regions (the F1 and F2 regions for forward primers and the
217 R1 and R2 regions for reverse primers), meaning that a wide range of amplicon lengths was expected
218 in the pre-amplification step using v1 (Fig. 3). Moreover, many Japanese Odonata species matched
219 more than two primer pairs (Table S1). In silico PCR demonstrated that the v1 primer set could
220 amplify not only all Japanese Odonata species but also 76.0% of Odonata 16S rRNA sequences in the
221 database (4050 of 5328 sequences; Fig. 4A). On the other hand, the v1 primer set may also anneal to a
222 relatively high proportion of sequences in non-Odonata taxa.

223 The v2 primer set was a modified version of v1; it consisted of two primer combinations that were
224 used separately to reduce non-Odonata amplification. Combination 1 included 4 forward primers and
225 7 reverse primers, whereas Combination 2 included 14 forward primers and 7 reverse primers. The

226 maximum difference in Tm values was 4.3 °C for Combination 1 and 5.8 °C for Combination 2. The
227 forward primers in v2 were selected from four distinct regions (the F1, F2, F3, and F4 regions; see
228 Fig. 3), while the reverse primers were selected from a single region (the R2 region). Unlike the v1
229 primer set, most species were expected to be amplified by only a single primer pair (Table S2). In
230 silico PCR suggested that the v2 primer set could amplify 69.4% of Odonata sequences in the
231 database (3961 sequences; Fig. 4B), including all Japanese Odonata species, and that in silico
232 amplification of non-Odonata taxa was lower than that observed with v1.

233 The v3 primer set consisted of 6 forward primers and 25 reverse primers. The maximum
234 difference in their Tm values was 5.1 °C. The forward and reverse primers were each selected from a
235 single region (the v3F and v3R regions, respectively; see Fig. 3), and therefore the amplicon lengths
236 were expected to be nearly constant (see Table S3 for primers corresponding to each species), with
237 only small variation due to insertions and deletions. For v3, specificity to Odonata species was
238 enhanced by the nucleotide at the 3′ end of the forward primers (i.e., adenine), because non-Odonata
239 taxa typically possess thymine at this site (Table S4). On the other hand, no Odonata-specific sites
240 were found in the candidate region for the reverse primers. The v3 primer set was expected to amplify
241 62.7% of Odonata sequences in the database (3340 sequences), including all Japanese Odonata
242 species, while amplification of non-Odonata sequences was almost completely suppressed (Fig. 4C).

243 The primer set for the first PCR consisted of one forward primer (5′-TGG AAG ACG AGA AGA
244 CCC TAT AGA-3′) and one reverse primer (5′-GAT TAC GCT GTT ATC CCT AAG GTA AC-3′).
245 These primers were selected from conserved regions, and therefore one of the regions overlapped with
246 insect-universal primers previously proposed. For example, the forward primer completely included
247 the region of the insect-universal primer MtInsect-16S (Takenaka et al. 2023). Therefore, the
248 first-PCR primer pair was not expected to completely prevent amplification of non-Odonata insects,
249 although it was expected to exclude sequences from non-insect taxa such as Crustacea, Mollusca, and
250 Annelida (Fig. 4D, E).

251

252 **Metabarcoding of field-collected samples**

253 Separate iSeq sequencing runs produced 2,295,839, 2,234,239, and 1,692,085 reads, and after quality
254 control, 2,157,854, 2,109,501, and 1,456,447 reads remained for v1, v2, and v3, respectively. The
255 numbers of reads assigned to Odonata were 1,846,430 (85.6%), 1,222,941 (58.0%), and 476,396
256 (32.7%). The numbers of Odonata reads obtained from PCR negative controls were 492 reads for v1,
257 137 reads for v2 (combined total from one PCR negative control and seven extraction blanks), and 3
258 reads for v3. The proportion of Odonata reads differed greatly among habitat types (Table 4). It was
259 generally high in samples from lentic environments (i.e., ponds, containers, and paddy fields). In
260 contrast, samples from lotic environments (i.e., streams) contained almost no Odonata reads (Table 4),
261 while the proportion of Ephemeroptera and Plecoptera reads was high for these samples (Tables S5–
262 S7). The total number of reads was also low in some lentic samples (e.g., Paddy 2 and Pond 1; Table
263 4), and the proportion of Odonata reads was generally low in such samples. The sequence data
264 obtained in this study are available from the DNA Data Bank of Japan (BioProject accession number
265 PRJDB40817).

266 We also examined the concordance between species detected by environmental DNA and the
267 observed presence/absence at the sampling sites. The species actually observed are listed in the
268 bottom row of Table 5. First, at sampling sites of Jinsekikogen in Hiroshima Prefecture (Paddies 1–7),
269 numerous adults and nymphs of *Sympetrum darwinianum* were observed (Fig. 1G, H), and this
270 species was detected via environmental DNA. At the Koishikawa Botanical Garden (Ponds 1–5),
271 adults of *Orthetrum albistylum*, *Orthetrum melania*, and *Pseudothemis zonata* (Fig. 1I) were
272 observed, and all of these were detected via environmental DNA. On the other hand, molted exuviae
273 of *Sympetrum baccha* were also found, but this species was not detected. At ponds of the Keio
274 Yochisha Elementary School (Ponds 6–8), adults of *Ceriagrion nipponicum*, *Crocothemis servilia*, *O.*
275 *albistylum*, *O. melania*, and *Rhyothemis fuliginosa* were frequently observed (Fig. 1J, K). Of these, all
276 except *R. fuliginosa* were detected via environmental DNA. At Okegayanuma, the endangered species
277 *L. angelina* was detected by all primer sets. Additionally, nymphs of species other than *L. angelina*

278 (*Anax nigrofasciatus*, *Ceriagrion melanurum*, and *C. servilia*) were observed in the containers, and all
279 except *A. nigrofasciatus* were detected via environmental DNA. In an upstream river on Mt. Tsukuba,
280 *Calopteryx cornelia* was frequently observed (Fig. 1L), but the species was not detected via
281 environmental DNA.
282
283 **Discussion**
284 **Performance of OdoCocktail-Japan primer sets**
285 The newly developed primer sets in this study were able to efficiently enrich Odonata reads
286 (hereafter, we refer to these primer sets as "OdoCocktail-Japan"). In previous studies that used
287 modified insect-universal primer sets to amplify Odonata DNA, the proportions of Odonata reads in
288 total reads were as low as 1–2% (Schwesig et al. 2025; Uche-Dike et al. 2026). Our study
289 demonstrates that the OdoCocktail-Japan primer sets can substantially improve eDNA metabarcoding
290 targeting Odonata: the mean proportions of Odonata reads were 61.3%, 54.3%, and 40.3% for v1, v2,
291 and v3, respectively. Moreover, these values may underestimate the performance of OdoCocktail-
292 Japan, because samples yielding 0–1% Odonata reads may simply have lacked detectable Odonata
293 eDNA or may have failed to amplify due to reasons unrelated to primer performance. When such
294 Odonata-undetectable samples were excluded, the proportions of Odonata reads increased markedly,
295 with the mean proportion for v1 reaching 95.4%. This high enrichment efficiency of
296 OdoCocktail-Japan is expected to reduce the cost of biodiversity assessments of Odonata using eDNA
297 metabarcoding.
298 OdoCocktail v1, v2, and v3 detected 17, 18, and 19 Odonata species, respectively, with ten or
299 more reads. All primer sets detected Odonata species with sufficiently high read numbers (e.g.,
300 >10,000) in many samples, although the detected species compositions differed slightly among the
301 primer sets, likely due to stochastic detection of low-abundance species (i.e., stochastic PCR
302 amplification) rather than differences in the performance of the primer sets. Such species dropout can
303 be mitigated by increasing the number of water samples or PCR replicates (Doi et al. 2019; Fukaya et

304 al. 2022). Therefore, an appropriate water-sampling strategy is also important for detecting the full
305 Odonata community at a survey site using OdoCocktail-Japan primer sets. Although we cannot
306 completely exclude the possibility that cross-contamination contributed to differences in detected
307 Odonata communities, such effects are likely negligible because the numbers of reads obtained from
308 negative controls were very low.

309 We detected Odonata species that are known to occur at the sampling sites. The most frequently
310 detected species was *S. darwinianum*, which inhabits and reproduces in paddy fields and wetlands
311 (Ozono, Kawashima, and Futahashi 2022), and this species was indeed detected from the paddy-field
312 samples (Table 5). Lotic odonates (*Lanthus fujiacus* and *Mnais costalis*) were also detected in some of
313 the paddy-field samples (Paddies 1–7), which were collected at the same site; however, nymphs of
314 these species do not generally occur in paddy fields. Because these species were also detected in the
315 sample collected from the irrigation channel flowing into the paddy fields (i.e., the Channel sample),
316 their DNA was likely transported into the paddy fields, as reported previously (Yamamoto et al.
317 2025). Many Odonata species that were observed when we collected samples from ponds and
318 containers were also detected in the eDNA samples. For the river samples, we detected a lotic
319 odonate, *Davidius fujiama*, from one sample, although the species differs from those observed at the
320 sampling site. These results suggest that the OdoCocktail-Japan primer sets are applicable to
321 field-collected eDNA samples. Notably, we detected *L. angelina*, a species designated as Critically
322 Endangered by both the IUCN and the Japanese Ministry of the Environment, indicating that the
323 OdoCocktail-Japan primer sets can be used for conservation purposes, such as identifying new
324 localities where endangered species occur. The OdoCocktail-Japan primers therefore provide a
325 low-cost and highly sensitive approach for assessing Odonata communities.

326

327 **Primer regions and specificity to Odonata**

328 Primers for eDNA metabarcoding are generally designed using a single pair of primer regions (i.e.,
329 one forward and one reverse region) and sometimes include degenerate bases to broaden taxonomic

330 coverage (Leese et al. 2021; Takenaka et al. 2023; Ushio et al. 2025; Thalinger et al. 2026). However,
331 this strategy showed the lowest efficiency among the OdoCocktail-Japan primer sets: samples
332 amplified with OdoCocktail v3 often showed intermediate proportions of Odonata reads (Table 4),
333 which was inconsistent with the performance predicted by in silico PCR (Fig. 4). Although
334 mismatches at the 3′ end of primers drastically reduce PCR efficiency, this effect may be mitigated
335 depending on the polymerase and buffer used (Bru, Martin-Laurent, and Philippot 2008; Huang et al.
336 2024). In particular, polymerases with 3′→5′ exonuclease activity can remove mismatches at the
337 primer 3′ end and may amplify non-target DNA (Anmarkrud et al. 2025). As a result, Ephemeroptera,
338 Plecoptera, Hemiptera, and Orthoptera were often co-amplified with Odonata in the same samples,
339 thereby decreasing the proportion of Odonata reads in v3. In contrast, for OdoCocktail v1 and v2,
340 primers were flexibly designed to perfectly match targeted sequences and avoid matching non-target
341 sequences by placing primer candidates at multiple distinct regions—four regions (two forward and
342 two reverse) for v1 and six regions (five forward and one reverse) for v2 (Fig. 3). Consequently, only
343 a few samples showed intermediate Odonata proportions (e.g., 20–80%), whereas in most samples
344 Odonata reads were either strongly enriched or hardly detected. This pattern suggests that v1 and v2
345 can efficiently amplify Odonata sequences when Odonata DNA is present in the sample. However,
346 both v1 and v2 may preferentially amplify other taxa when Odonata DNA is absent or present only in
347 very low concentrations. For example, some samples yielded almost no Odonata reads despite high
348 total read counts (e.g., paddy-9 with v1, container-2 with v2, and river samples with both v1 and v2;
349 Table 4). In these samples, reads from Anostraca (paddy-9 with v1), Diplostraca (container-2 with
350 v2), and both Ephemeroptera and Plecoptera (river samples with v1 and v2) were abundant (Tables
351 S5–S7). These results indicate that OdoCocktail v1 and v2 can amplify alternative taxa when Odonata
352 DNA is absent or extremely rare. Because Odonata often occur at lower abundance than
353 Ephemeroptera and Plecoptera in lotic environments, Odonata DNA is more difficult to detect in river
354 samples. To minimize primer combinations that could amplify Ephemeroptera and Plecoptera, the v2
355 primer set was divided into two groups; however, the effect of separate use appeared to be limited,

356 even though one Odonata species was detected in a river sample (river-2). Thus, further improvement
357 of OdoCocktail-Japan is needed for reliable detection of Odonata in lotic environments.
358

359 **Amplicon length of targeted sequences**

360 Because eDNA analysis usually targets DNA molecules that are degraded in the environment, shorter
361 amplicons generally show higher detection efficiency than longer ones (Jo et al. 2017; Wei, Nakajima,
362 and Tobino 2018). Considering this, detection efficiency may differ among species when OdoCocktail
363 v1 or v2 is used, because the amplicon lengths generated by these primer sets differ by up to ~80 bp
364 (387–406 bp for the F1–R2 combination and 308–327 bp for the F2–R1 combination; Fig. 3). For v2,
365 the majority of species are amplified using the F1–R2 combination, so the variation in amplicon
366 length is limited. In contrast, for v1 the variation in amplicon lengths among species is substantial.
367 Nevertheless, OdoCocktail v1 successfully detected multiple species whose pre-amplification
368 amplicons likely differed in length. For example, *P. zonata* perfectly matches F1a as the forward
369 primer and R1b, R2j, and R2l as reverse primers, resulting in the shortest possible amplicon length of
370 375 bp. This species was detected in a sample together with *C. melanurum* (shortest amplicon 324
371 bp), *R. fuliginosa* (313 bp), and *O. albistylum* (314 bp). Two possible explanations exist for this result.
372 One is that a difference of 55 bp in amplicon length may not substantially affect DNA degradation or
373 PCR efficiency. The other is that F2 primers may have contributed to the amplification of *P. zonata*.
374 Although the F2 primers have a mismatch at the fourth base from the 3′ end for *P. zonata*, such a
375 mismatch may reduce—but not completely prevent—PCR amplification (Bru, Martin-Laurent, and
376 Philippot 2008; Huang et al. 2024). In addition, reaction buffer composition and annealing
377 temperature may mitigate the effect of mismatches (Ishii and Fukui 2001; Stadhouders et al. 2010). In
378 either case, we were able to detect species whose pre-amplification amplicons were expected to be
379 longer. However, to correctly interpret data obtained using OdoCocktail-Japan metabarcoding, it will
380 be necessary to address the effect of amplicon length on species-specific detection efficiency.
381

382 **Expansion of availability outside Japan**

383 Although the OdoCocktail-Japan primers are not specifically designed to detect Odonata species
384 outside Japan, it is possible to modify the primer sets to target species occurring in other geographic
385 regions. For example, of the 102 Odonata species reported from Korea (Yoon 2023), at least 86
386 species (84%) also occur in Japan. Moreover, 16S rRNA sequences are available in public DNA
387 databases for 14 of the 16 Korean species that do not occur in Japan. Therefore, an
388 "OdoCocktail-Korea" set could be developed by adding primers to amplify these 14 Korean-specific
389 species and removing primers unnecessary for the 86 shared species. In Austria, 16S rRNA sequences
390 are available for 75 of the 78 known Odonata species (Zangl et al. 2025). Although only 21 Austrian
391 species (27%) occur in Japan, 78% of the genera are shared between the two countries. Given that
392 congeneric species can generally be amplified by the same primer pairs, primer sets to detect Austrian
393 Odonata species could be developed based on the OdoCocktail-Japan design. Thus, OdoCocktail can
394 be modified to detect Odonata fauna in specific countries or regions. Such modified primer sets would
395 require validation by confirming that the pre-amplification products are compatible with the inner
396 primers of the first PCR and by testing with field samples and/or mock DNA samples.
397
398 **Conclusion**

399 Developing metabarcoding primer sets (e.g., Miya et al. 2015; Sakata et al. 2022) for eDNA analysis
400 has facilitated the assessment and monitoring of biodiversity in aquatic organisms such as fish and
401 amphibians (Miya 2022; Takahashi et al. 2023; Broadhurst et al. 2025; Takenaka et al. 2026). eDNA
402 metabarcoding primers have also been developed for insects, expanding opportunities to assess
403 aquatic insect diversity (Leese et al. 2021; Takenaka et al. 2023), but incomplete reference databases
404 often hinder these efforts (Richardson et al. 2018; Takahashi et al. 2023; Alvarado-Robledo et al.
405 2024; Plewnia et al. 2026). OdoCocktail-Japan addresses this issue by targeting 16S rRNA sequences,
406 which are available for all Japanese Odonata species except *Diplacodes bipunctatus*. In addition, the
407 OdoCocktail-Japan primer sets efficiently enrich Odonata reads, enabling cost-effective eDNA

408 monitoring of Odonata communities. We believe that metabarcoding using the OdoCocktail-Japan
409 primer sets can help to assess the efficiency of conservation actions such as biodiversity-friendly rice
410 farming (Giuliano and Bogliani 2019) and citizen science targeting Odonata species. Given its high
411 enrichment performance and low cost, the OdoCocktail v1 primer set currently offers the most
412 efficient detection of Odonata species in Japan. In eDNA-based biodiversity assessment, not only
413 primer selection but also the selection of water-sampling sites and the number of sampling replicates
414 are important factors influencing detection efficiency (Fukaya et al. 2022). However, no standardized
415 protocols exist for monitoring Odonata fauna. The OdoCocktail-Japan primers can help address these
416 issues. In the supplementary materials, we describe the procedures for metabarcoding library
417 preparation and sequence data analysis, with the aim of making eDNA-based monitoring of Odonata
418 species more accessible.
419

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430

431 **Figure legends**

432 Figure 1. **Sampling sites and species encountered during sampling.** (A) Photograph of the paddy
433 field where the Paddy 1 sample was collected (Jinsekikogen, Hiroshima). (B) A small fountain pond

434 where the Pond 2 and 3 samples were collected (Koishikawa Botanical Garden, Tokyo). The Pond 1
435 sample was collected from another small fountain pond. (C) A garden pond where the Pond 4 and 5
436 samples were collected (Koishikawa Botanical Garden, Tokyo). (D) A biotope pond where the Pond
437 6–8 samples were collected (Keio Yochisha Elementary School, Tokyo). (E) Containers used for the
438 Container 1–5 samples (Okegayanuma, Shizuoka). (F) The stream where the River 1–4 samples were
439 collected (Mt. Tsukuba, Ibaraki). (G) Immature adults of *Sympetrum darwinianum* and (H)
440 *Sympetrum* nymphs including *S. darwinianum* at Jinsekikogen; (I) *Pseudothemis zonata* at
441 Koishikawa Botanical Garden; (J) *Crocothemis servilia*, (K) *Orthetrum albistylum* and *Rhyothemis*
442 *fuliginosa* at Keio Yochisha Elementary School (photos by T. Suguro); and (L) *Calopteryx cornelia* at
443 Mt. Tsukuba.
444
445 Figure 2. **Schematic diagram of sample handling and replication procedures.** Gray circles indicate
446 samples and replicates, and small open circles on lines indicates the points where replicates or
447 samples were pooled.
448
449 Figure 3. **Positions of primer regions.** Arrows indicate the locations of primer regions and their
450 position in the alignment of Odonata 16S rRNA sequences. The histogram at the top shows the
451 proportion of the majority base at each nucleotide position.
452
453 Figure 4. **Proportion of sequences amplified in in silico PCR.** Upper panels show the results of the
454 pre-amplification step using (A) version 1, (B) version 2, and (C) version 3 primer sets. Lower panels
455 show the results of first PCR. The templates for the first PCR correspond to the DNA amplified by
456 (D) version 1, (E) version 2, and (F) version 3 primer sets.
457

458 **References**

459 Alvarado-Robledo, E. J., I. M. Hernández-Velázquez, K. Guillén-Navarro, E. Diego-García, E. Zarza,
460 and J. A. Zamora-Briseño. 2024. "Metabarcoding: Opportunities for Accelerating Monitoring and
461 Understanding Insect Tropical Biodiversity." *Journal of Insect Conservation* **28**, no. 4: 589–604.
462 https://doi.org/10.1007/s10841-024-00584-1.

463 Anmarkrud, J. A., F. dos Anjos Santa Rosa, L. Thorbek, A. Schrøder-Nielsen, S. M. Sviggum, J. S.
464 Ready, H. J. de Boer, and Q. Mauvisseau. 2025. "eDNA Replicates, Polymerase and Amplicon
465 Size Impact Inference of Richness Across Habitats." *Environmental DNA* **7**, no. 3: e70095.
466 https://doi.org/10.1002/edn3.70095.

467 Arthington, A. H. 2021. "Grand Challenges to Support the Freshwater Biodiversity Emergency
468 Recovery Plan." *Frontiers in Environmental Science*, no. 9: 664313.
469 https://doi.org/10.3389/fenvs.2021.664313.

470 Baba, Y. G., Y. Kusumoto, and K. Tanaka. 2019. "Positive Effect of Environmentally Friendly
471 Farming on Paddy Field Odonate Assemblages at a Small Landscape Scale." *Journal of Insect*
472 *Conservation* **23**, no. 3: 467–474. https://doi.org/10.1007/s10841-019-00132-2.

473 Boyer, F., C. Mercier, A. Bonin, Y. Le Bras, P. Taberlet, and E. Coissac. 2016. "Obitools: A Unix-
474 Inspired Software Package for DNA Metabarcoding." *Molecular Ecology Resources* **16**, no. 1:
475 176–182. https://doi.org/10.1111/1755-0998.12428.

476 Broadhurst, H. A., N. G. Sales, R. Raynor, C. Howe, E. Ochu, X. Lambin, C. S. Sutherland, and A. D.
477 McDevitt. 2025. "From Water to Land: A Review on the Applications of Environmental DNA
478 and Invertebrate-Derived DNA for Monitoring Terrestrial and Semi-Aquatic Mammals." *Mammal*
479 *Review* **55**, no. 4: e70006. https://doi.org/10.1111/mam.70006.

480 Bru, D., F. Martin-Laurent, and L. Philippot. 2008. "Quantification of the Detrimental Effect of a
481 Single Primer-Template Mismatch by Real-Time PCR Using the 16S rRNA Gene as an

482 Example." *Applied and Environmental Microbiology* **74**, no. 5: 1660–1663.
483 https://doi.org/10.1128/AEM.02403-07.
484 Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016.
485 "DADA2: High-Resolution Sample Inference from Illumina Amplicon Data." *Nature Methods*
486 **13**, no. 7: 581–583. https://doi.org/10.1038/nmeth.3869.
487 Camacho, C., G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, and T. L. Madden. 2009.
488 "BLAST+: Architecture and Applications." *BMC Bioinformatics* **10**, no. 1: 421.
489 https://doi.org/10.1186/1471-2105-10-421.
490 Chen, S., Y. Zhou, Y. Chen, and J. Gu. 2018. "fastp: an ultra-fast all-in-one FASTQ preprocessor."
491 *Bioinformatics* **34**, no. 17: i884–i890. https://doi.org/10.1093/bioinformatics/bty560.
492 Clarke, S. J., E. Long, J. Biggs, K. Bruce, A. Weatherby, L. R. Harper, and R. S. Hails. 2023. "Co-
493 Design of a Citizen Science Study: Unlocking the Potential of eDNA for Volunteer Freshwater
494 Monitoring." *Ecological Solutions and Evidence* **4**, no. 3: e12273. https://doi.org/10.1002/2688-
495 8319.12273.
496 Doi, H., K. Fukaya, S. Oka, K. Sato, M. Kondoh, and M. Miya. 2019. "Evaluation of Detection
497 Probabilities at the Water-Filtering and Initial PCR Steps in Environmental DNA Metabarcoding
498 Using a Multispecies Site Occupancy Model." *Scientific Reports* **9**, no. 1: 3581.
499 https://doi.org/10.1038/s41598-019-40233-1.
500 Fan, G., S. Qin, and C. Hu. 2025. "Retrospection of Research on Dragonfly and Damselfly (Odonata)
501 During Past Fifty Years: A Bibliometric Review." *Insects* **16**, no. 9: 945.
502 https://doi.org/10.3390/insects16090945.
503 Fukaya, K., N. I. Kondo, S. S. Matsuzaki, and T. Kadoya. 2022. "Multispecies Site Occupancy
504 Modelling and Study Design for Spatially Replicated Environmental DNA Metabarcoding."
505 *Methods in Ecology and Evolution* **13**, no. 1: 183–193. https://doi.org/10.1111/2041-210X.13732.

506 Giuliano, D., and G. Bogliani. 2019. “Odonata in Rice Agroecosystems: Testing Good Practices for
507 Their Conservation.” *Agriculture, Ecosystems & Environment* **275**, 65–72.
508 https://doi.org/10.1016/j.agee.2019.01.009.
509 Hoshina, H. 2022. “The Mythology of Insect-Loving Japan” *Insects* **13**. No. 3:234.
510 https://doi.org/10.3390/insects13030234.
511 Huang, K., J. Zhang, J. Li, H. Qiu, L. Wei, Y. Yang, and C. Wang. 2024. “Exploring the Impact of
512 Primer–Template Mismatches on PCR Performance of DNA Polymerases Varying in
513 Proofreading Activity.” *Genes* **15**, no. 2: 215. https://doi.org/10.3390/genes15020215.
514 Ishii, K., and M. Fukui. 2001. “Optimization of Annealing Temperature to Reduce Bias Caused by a
515 Primer Mismatch in Multitemplate PCR.” *Applied and Environmental Microbiology* **67**, no. 8:
516 3753–3755. https://doi.org/10.1128/AEM.67.8.3753-3755.2001.
517 Jo, T., H. Murakami, R. Masuda, M. K. Sakata, S. Yamamoto, and T. Minamoto. 2017. “Rapid
518 Degradation of Longer DNA Fragments Enables the Improved Estimation of Distribution and
519 Biomass Using Environmental DNA.” *Molecular Ecology Resources* **17**, no. 6: e25–33.
520 https://doi.org/https://doi.org/10.1111/1755-0998.12685.
521 Katayama, N., and Y. G. Baba. 2020. “Measuring Artistic Inspiration Drawn from Ecosystems and
522 Biodiversity: A Case Study of Old Children’s Songs in Japan.” *Ecosystem Services* **43**, 101116.
523 https://doi.org/10.1016/j.ecoser.2020.101116.
524 Katoh, K., and D. M. Standley. 2013. “MAFFT Multiple Sequence Alignment Software Version 7:
525 Improvements in Performance and Usability.” *Molecular Biology and Evolution* **30**, no. 4: 772–
526 780. https://doi.org/10.1093/molbev/mst010.
527 Lee Foote, A., and C. L. Rice Hornung. 2005. “Odonates as Biological Indicators of Grazing Effects
528 on Canadian Prairie Wetlands.” *Ecological Entomology* **30**, no. 3: 273–283.
529 https://doi.org/10.1111/j.0307-6946.2005.00701.x.
530 Leese, F., M. Sander, D. Buchner, V. Elbrecht, P. Haase, and V. M. A. Zizka. 2021. “Improved
531 Freshwater Macroinvertebrate Detection from Environmental DNA Through Minimized

532 Nontarget Amplification." *Environmental DNA* **3**, no. 1: 261–276.
533 https://doi.org/10.1002/edn3.177.
534 Liang, L., Y. Wang, X. Lu, Y. Feng, X. Mo, D. Zhang, and C. Li. 2026. "LiBird: A New Universal
535 Primer Enhancing Avian Environmental DNA Metabarcoding." *Avian Research* **17**, no. 2:
536 100349. https://doi.org/10.1016/j.avrs.2026.100349.
537 Miguel, T. B., J. M. B. Oliveira-Junior, R. Ligeiro, and L. Juen. 2017. "Odonata (Insecta) as a Tool
538 for the Biomonitoring of Environmental Quality." *Ecological Indicators* **81**, 555–566.
539 https://doi.org/10.1016/j.ecolind.2017.06.010.
540 Minamoto, T., M. Miya, T. Sado, S. Seino, H. Doi, M. Kondoh, K. Nakamura, et al. 2021. "An
541 Illustrated Manual for Environmental DNA Research: Water Sampling Guidelines and
542 Experimental Protocols." *Environmental DNA* **3**, no. 1: 8–13. https://doi.org/10.1002/edn3.121.
543 Miya, M. 2022. "Environmental DNA Metabarcoding: A Novel Method for Biodiversity Monitoring
544 of Marine Fish Communities." *Annual Review of Marine Science* **14**, 161–185.
545 https://doi.org/10.1146/annurev-marine-041421-082251.
546 Miya, M., Y. Sato, T. Fukunaga, T. Sado, J. Y. Poulsen, K. Sato, T. Minamoto, et al. 2015. "MiFish, a
547 Set of Universal PCR Primers for Metabarcoding Environmental DNA from Fishes: Detection of
548 More Than 230 Subtropical Marine Species." *Royal Society Open Science* **2**, no. 7: 150088.
549 https://doi.org/10.1098/rsos.150088.
550 Okonechnikov, K., O. Golosova, M. Fursov, and the UGENE team. 2012. "Unipro UGENE: A
551 Unified Bioinformatics Toolkit." *Bioinformatics* **28**, no. 8: 1166–1167.
552 https://doi.org/10.1093/bioinformatics/bts091.
553 Ozono, A., I. Kawashima, and R. Futahashi. 2022. *Dragonflies in Japan*. Second ed. Bun-ichi
554 Co.,Ltd.
555 Pascher, K., V. Švara, and M. Jungmeier. 2022. "Environmental DNA-Based Methods in Biodiversity
556 Monitoring of Protected Areas: Application Range, Limitations, and Needs." *Diversity* **14**, no. 6:
557 463. https://doi.org/10.3390/d14060463.

558 Plewnia, A., T. Hildwein, A. B. Quezada Riera, A. Terán-Valdez, A. J. Crawford, C. Heine, D.
559 Franco-Mena, et al. 2026. "Environmental DNA Metabarcoding Facilitates Integrative
560 Conservation Assessments and Species Rediscoveries in Tropical Biodiversity Hotspots."
561 *Scientific Reports* **16**, no. 1: 8150. https://doi.org/10.1038/s41598-026-41937-x.
562 Priyadarshana, T. S., and Slade, E. M. 2023. "A meta-analysis reveals that dragonflies and damselflies
563 can provide effective biological control of mosquitoes." *Journal of Animal Ecology* **92**, no.
564 8:1589–1600. https://doi.org/10.1111/1365-2656.13965.
565 R Core Team. 2024. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R
566 Foundation for Statistical Computing. https://www.R-project.org/.
567 Richardson, R. T., J. Bengtsson-Palme, M. M. Gardiner, and R. M. Johnson. 2018. "A Reference
568 Cytochrome C Oxidase Subunit I Database Curated for Hierarchical Classification of Arthropod
569 Metabarcoding Data." *PeerJ* **6**, e5126. https://doi.org/10.7717/peerj.5126.
570 Rocha-Ortega, M., P. Rodríguez, and A. Córdoba-Aguilar. 2019. "Can Dragonfly and Damselfly
571 Communities Be Used as Bioindicators of Land Use Intensification?" *Ecological Indicators* **107**,
572 105553. https://doi.org/10.1016/j.ecolind.2019.105553.
573 Sakata, M. K., M. U. Kawata, A. Kurabayashi, T. Kurita, M. Nakamura, T. Shirako, R. Kakehashi, et
574 al. 2022. "Development and Evaluation of PCR Primers for Environmental DNA (eDNA)
575 Metabarcoding of Amphibia." *Metabarcoding and Metagenomics* **6**, e76534.
576 https://doi.org/10.3897/mbmg.6.76534.
577 Sayer, C. A., E. Fernando, R. R. Jimenez, N. B. W. Macfarlane, G. Rapacciuolo, M. Böhm, T. M.
578 Brooks, et al. 2025. "One-Quarter of Freshwater Fauna Threatened with Extinction." *Nature* **638**,
579 no. 8049: 138–145. https://doi.org/10.1038/s41586-024-08375-z.
580 Schwesig, K., V. Zizka, C. Scherber, and N. Hölzel. 2025. "Comparing eDNA and Transect Methods
581 for Aquatic Biodiversity Assessment in Lakes and Ponds." *Molecular Ecology Resources* **25**, no.
582 3: e14060. https://doi.org/10.1111/1755-0998.14060.

583 Shen, W., and H. Ren. 2021. "TaxonKit: A Practical and Efficient NCBI Taxonomy Toolkit." *Journal
584 of Genetics and Genomics* **48**, no. 9: 844–850. https://doi.org/10.1016/j.jgg.2021.03.006.
585 Shen, W., S. Le, Y. Li, F. Hu. 2016. "SeqKit: A Cross-Platform and Ultrafast Toolkit for FASTA/Q
586 File Manipulation." *PLOS ONE* **11**, no. 10: e0163962.
587 https://doi.org/10.1371/journal.pone.0163962.
588 Šigutová, H., J. Šipoš, and A. Dolný. 2019. "A Novel Approach Involving the Use of Odonata as
589 Indicators of Tropical Forest Degradation: When Family Matters." *Ecological Indicators* **104**,
590 229–236. https://doi.org/10.1016/j.ecolind.2019.05.001.
591 Stadhouders, R., S. D. Pas, J. Anber, J. Voermans, T. H. M. Mes, and M. Schutten. 2010. "The Effect
592 of Primer-Template Mismatches on the Detection and Quantification of Nucleic Acids Using the
593 5′ Nuclease Assay." *The Journal of Molecular Diagnostics* **12**, no. 1: 109–117.
594 https://doi.org/10.2353/jmoldx.2010.090035.
595 Su, N. 2022. "Insects in Japanese Culture, and One That Saved the Country" *American Entomologist*
596 **68**, no. 1:52–58. https://doi.org/10.1093/ae/tmac010.
597 Takahashi, M., M. Saccò, J. H. Kestel, G. Nester, M. A. Campbell, M. van der Heyde, M. J.
598 Heydenrych, et al. 2023. "Aquatic Environmental DNA: A Review of the Macro-Organismal
599 Biomonitoring Revolution." *Science of The Total Environment* **873**, 162322.
600 https://doi.org/10.1016/j.scitotenv.2023.162322.
601 Takenaka, M., K. Yano, T. Suzuki, and K. Tojo. 2023. "Development of Novel PCR Primer Sets for
602 DNA Barcoding of Aquatic Insects, and the Discovery of Some Cryptic Species." *Limnology* **24**,
603 no. 2: 121–136. https://doi.org/10.1007/s10201-022-00710-5.
604 Takenaka, M., N. Uchida, Y. Hasebe, K. Yano, and G. Ueki. 2026. "Environmental DNA
605 Metabarcoding for Insect Biodiversity Surveys: Current Status and Future Prospects for Potential
606 Applications in Highly Diverse Insect Taxa." *Entomological Science* **29**, no. 1: e12616.
607 https://doi.org/10.1111/ens.12616.

608 Takeuchi, A., T. Sado, R. O. Gotoh, S. Watanabe, K. Tsukamoto, and M. Miya. 2019. "New PCR
609 Primers for Metabarcoding Environmental DNA from Freshwater Eels, Genus *Anguilla*."
610 *Scientific Reports* **9**, no. 1: 7977. https://doi.org/10.1038/s41598-019-44402-0.
611 Thalinger, B., R. Empey, M. Cowperthwaite, K. Coveny, and D. Steinke. 2026. "BirT: A Novel
612 Primer Pair for Avian Environmental DNA Metabarcoding." *Environmental DNA* **8**, no. 1:
613 e70255. https://doi.org/10.1002/edn3.70255.
614 Uche-Dike, R., E. Tolman, C. Benischek, M. Schneider, M. K. Kohli, J. Bush, P. B. Frandsen, et al.
615 2026. "Integrating eDNA and Community Science to Monitor Urban Odonata Diversity." *Insect*
616 *Conservation and Diversity* **19**, no. 1: 84–92. https://doi.org/10.1111/icad.70006.
617 Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B. C. Faircloth, M. Remm, and S. G. Rozen. 2012.
618 "Primer3—New Capabilities and Interfaces." *Nucleic Acids Research* **40**, no. 15: e115–115.
619 https://doi.org/10.1093/nar/gks596.
620 Ushio, M., K. Murata, T. Sado, I. Nishiumi, M. Takeshita, W. Iwasaki, and M. Miya. 2018.
621 "Demonstration of the Potential of Environmental DNA as a Tool for the Detection of Avian
622 Species." *Scientific Reports* **8**, no. 1: 4493. https://doi.org/10.1038/s41598-018-22817-5.
623 Ushio, M., S. Ozawa, S. Oka, T. Sado, R. O. Kisero, L. Porter, E. Matrai, and M. Miya. 2025. "*μ*Ceta:
624 A Set of Cetacean-Specific Primers for Environmental DNA Metabarcoding with Minimal
625 Amplification of Non-Target Vertebrates." *Environmental DNA* **7**, no. 5: e70193.
626 https://doi.org/10.1002/edn3.70193.
627 Valentini, A., P. Taberlet, C. Miaud, R. Civade, J. Herder, P. F. Thomsen, E. Bellemain, et al. 2016.
628 "Next-Generation Monitoring of Aquatic Biodiversity Using Environmental DNA
629 Metabarcoding." *Molecular Ecology* **25**, no. 4: 929–942. https://doi.org/10.1111/mec.13428.
630 Wei, N., F. Nakajima, and T. Tobino. 2018. "Effects of Treated Sample Weight and DNA Marker
631 Length on Sediment eDNA Based Detection of a Benthic Invertebrate." *Ecological Indicators* **93**,
632 267–273. https://doi.org/10.1016/j.ecolind.2018.04.063.

633 Yamamoto, S., N. Katayama, J. Yamasako, K. Ito, S. Okubo, and Y. G. Baba. 2025. "Optimal
634 Environmental DNA Sampling Strategy for Comprehensively Assessing Biodiversity in Rice
635 Paddy Fields." *Environmental DNA* **7**, no. 6: e70224. https://doi.org/10.1002/edn3.70224.
636 Yoon, S. 2023. "The Integrated Dataset for Occurrence of Odonata Species in South Korea." *GEO
637 DATA* **5**, no. 2: 77–86. https://doi.org/10.22761/GD.2023.0013.
638 Zangl, L., I. Fischer, M. Sittenthaler, A. Chovanec, P. Gros, W. Holzinger, G. Kunz, et al. 2025. "A
639 DNA Barcode Inventory of Austrian Dragonfly and Damselfly (Insecta: Odonata) Species."
640 *Insects* **16**, no. 10: 1056. https://doi.org/10.3390/insects16101056.
641

642 **Data Accessibility**

643 Raw sequence reads have been deposited in DDBJ (BioProject PRJDB40817). Custom scripts are
644 available in the Supplementary Materials.

645 **Benefit-Sharing**

646 Benefits Generated: Benefits from this research accrue from the sharing of our data on public
647 databases as described above.

648 **Author Contributions**

649 S.Y. and Y.G.B. designed the research. S.Y., N.K., J.Y., K.I., and Y.G.B. collected field samples.
650 S.Y. performed molecular experiments, contributed analytical tools, analyzed data and wrote the first
651 draft. Y.G.B. acquired funding. All authors reviewed and edited manuscript.

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653

654

655 Figure 1. **Sampling sites and species encountered during sampling.** (A) Photograph of the paddy
656 field where the Paddy 1 sample was collected (Jinsekikogen, Hiroshima). (B) A small fountain pond
657 where the Pond 2 and 3 samples were collected (Koishikawa Botanical Garden, Tokyo). The Pond 1
658 sample was collected from another small fountain pond. (C) A garden pond where the Pond 4 and 5
659 samples were collected (Koishikawa Botanical Garden, Tokyo). (D) A biotope pond where the Pond
660 6–8 samples were collected (Keio Yochisha Elementary School, Tokyo). (E) Containers used for the
661 Container 1–5 samples (Okegayanuma, Shizuoka). (F) The stream where the River 1–4 samples were

662 collected (Mt. Tsukuba, Ibaraki). (G) Immature adults of *Sympetrum darwinianum* and (H)

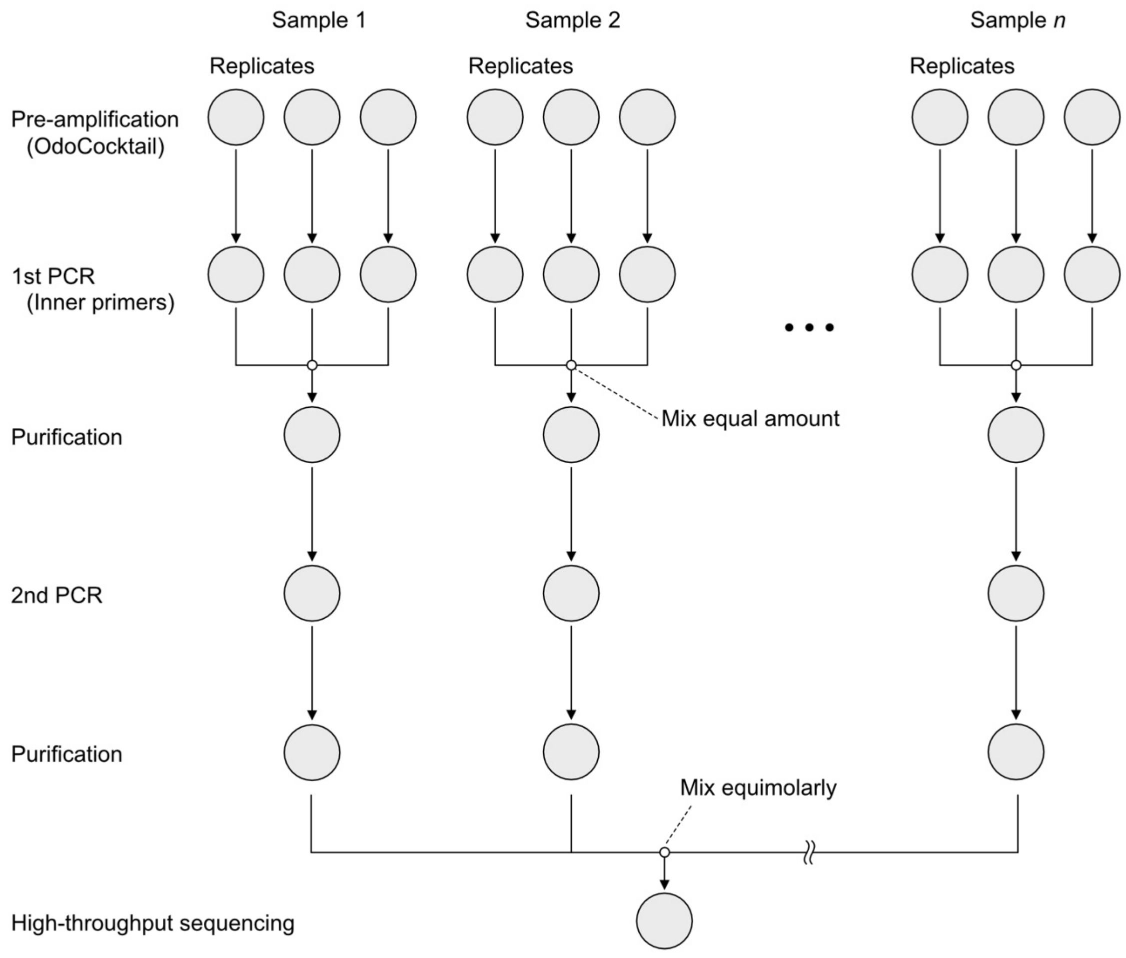
663 *Sympetrum* nymphs including *S. darwinianum* at Jinsekikogen; (I) *Pseudothemis zonata* at

664 Koishikawa Botanical Garden; (J) *Crocothemis servilia*, (K) *Orthetrum albistylum* and *Rhyothemis*

665 *fuliginosa* at Keio Yochisha Elementary School (photos by T. Suguro); and (L) *Calopteryx cornelia* at

666 Mt. Tsukuba.

667



668

669 Figure 2. **Schematic diagram of sample handling and replication procedures.** Gray circles indicate

670 samples and replicates, and small open circles on lines indicates the points where replicates or

671 samples were pooled.

672

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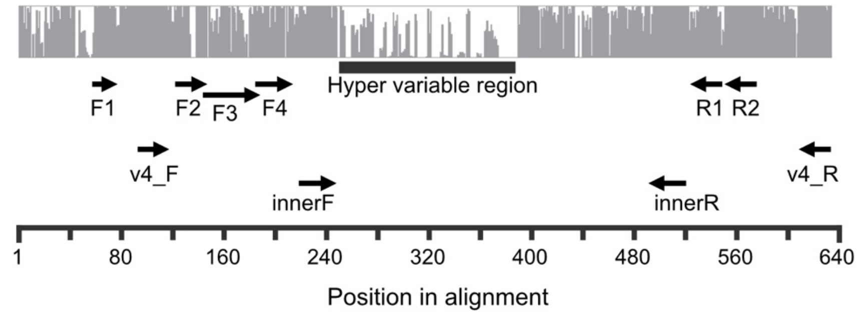


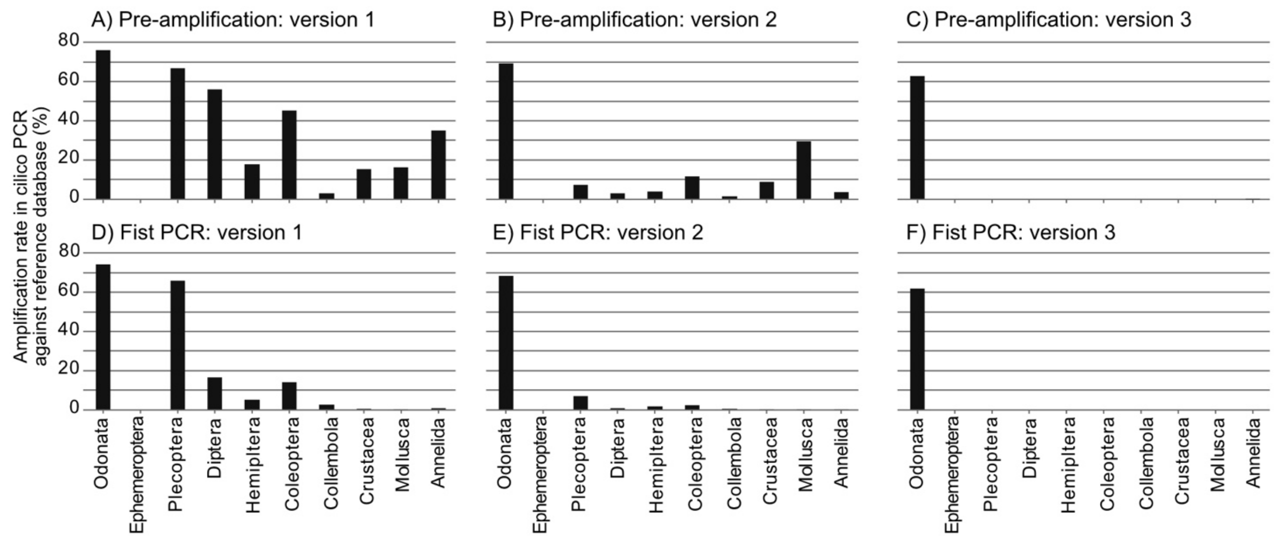
674

675 Figure 3. **Positions of primer regions.** Arrows indicate the locations of primer regions and their

676 position in the alignment of Odonata 16S rRNA sequences. The histogram at the top shows the

677 proportion of the majority base at each nucleotide position.

678



679

680

681 Figure 4. **Proportion of sequences amplified in in silico PCR.** Upper panels show the results of the

682 pre-amplification step using (A) version 1, (B) version 2, and (C) version 3 primer sets. Lower panels

683 show the results of first PCR. The templates for the first PCR correspond to the DNA amplified by

684 (D) version 1, (E) version 2, and (F) version 3 primer sets.

Table 1. Samples used in this study.

ID	Sample name	Sample origin	Sampling location	Habitat type	Filtered water ammount	Sampling date
Paddy-1	HIJ230706-TA-01	Organic rice paddy field	Jinsekikogen, Hiroshima	Lentic	350	July 6, 2023
Paddy-2	HIJ230706-TA-02	Organic rice paddy field	Jinsekikogen, Hiroshima	Lentic	100	July 6, 2023
Paddy-3	HIJ230706-TA-03	Organic rice paddy field	Jinsekikogen, Hiroshima	Lentic	250	July 6, 2023
Paddy-4	HIJ230706-TA-03-hiyose	Small channel within rice paddy field	Jinsekikogen, Hiroshima	Lentic	150	July 6, 2023
Paddy-5	HIJ230706-Con01	Conventional rice paddy field	Jinsekikogen, Hiroshima	Lentic	200	July 6, 2023
Paddy-6	HIJ230706-Con02	Conventional rice paddy field	Jinsekikogen, Hiroshima	Lentic	400	July 6, 2023
Paddy-7	HIJ230706-Con03	Conventional rice paddy field	Jinsekikogen, Hiroshima	Lentic	500	July 6, 2023
Paddy-8	eKMK220602-02-6	Organic rice paddy field	Kaminokawa, Tochigi	Lentic	500	June 2, 2022
Paddy-9	eKMK220602-03-6	Conventional rice paddy field	Kaminokawa, Tochigi	Lentic	500	June 2, 2022
Paddy-10	eKMK220602-08-6	Organic rice paddy field	Kaminokawa, Tochigi	Lentic	500	June 2, 2022
Pond-1	eKoBG230711-1	Small fountain pond (South)	Koishikawa botanical garden, Bunkyo, Tokyo	Lentic	240	July 11, 2023
Pond-2	eKoBG230711-2	Small fountain pond (North)	Koishikawa botanical garden, Bunkyo, Tokyo	Lentic	500	July 11, 2023
Pond-3	eKoBG230711-3	Small fountain pond (North)	Koishikawa botanical garden, Bunkyo, Tokyo	Lentic	500	July 11, 2023
Pond-4	eKoBG230711-4	Garden pond	Koishikawa botanical garden, Bunkyo, Tokyo	Lentic	500	July 11, 2023
Pond-5	eKoBG230711-5	Garden pond	Koishikawa botanical garden, Bunkyo, Tokyo	Lentic	300	July 11, 2023
Pond-6	eKOY230728-1	Biotope pond	Keio Yochisha Elementary School, Shibuya, Tokyo	Lentic	500	July 28, 2023
Pond-7	eKOY230728-2	Biotope pond	Keio Yochisha Elementary School, Shibuya, Tokyo	Lentic	500	July 28, 2023
Pond-8	eKOY230728-3	Biotope pond	Keio Yochisha Elementary School, Shibuya, Tokyo	Lentic	500	July 28, 2023
Container-1	eOKG230808-1	Insect rearing container	Okegayanuma, Shizuoka	Lentic	270	August 8, 2023
Container-2	eOKG230808-2	Insect rearing container	Okegayanuma, Shizuoka	Lentic	35	August 8, 2023
Container-3	eOKG230808-3	Insect rearing container	Okegayanuma, Shizuoka	Lentic	140	August 8, 2023
Container-4	eOKG230808-4	Insect rearing container	Okegayanuma, Shizuoka	Lentic	40	August 8, 2023
Container-5	eOKG230808-5	Insect rearing container	Okegayanuma, Shizuoka	Lentic	90	August 8, 2023
Channel	HIJ230706-channel	Small irrigation channel	Jinsekikogen, Hiroshima	Lotic	500	July 6, 2023
River-1	eTKBM230714-1-B	Upstream river	Mt. Tsukuba, Ibaraki	Lotic	500	July 14, 2023
River-2	eTKBM230714-1-Y	Upstream river	Mt. Tsukuba, Ibaraki	Lotic	500	July 14, 2023
River-3	eYKBM230714-2-B	Upstream river	Mt. Tsukuba, Ibaraki	Lotic	500	July 14, 2023
River-4	eTKBM230714-2-Y	Upstream river	Mt. Tsukuba, Ibaraki	Lotic	500	July 14, 2023

688 Table 2. PCR conditions used in the study.

	Pre-amplification				1st PCR				2nd PCR			
Primer set	Polymerase	Annering	cycle	replicate	Polymerase	Annering	cycle	replicate	Polymerase	Annering	cycle	replicate
v1	KOD One	58ºC	20	3	KOD One	58ºC	20	3	KAPA HiFi HotStart	72ºC	15	1
v2	KOD One	58ºC	20	3	KOD One	58ºC	20	3	KAPA HiFi HotStart	72ºC	15	1
v3	KOD One	54ºC	20	3	KOD One	58ºC	20	3	KAPA HiFi HotStart	72ºC	15	1

689

690 Table 3. Primers of OdoCocktail-Japan.

OdoCocktail version	Primer name	Sequence (5' -> 3')	Length (bp)	GC content (%)
v1	Forward primer			
	F1a	TTTAACGGCCGCGGTATAC	19	52.6
	F1b	TTTAACGGCCGCGGTATAT	19	47.4
	F1d	TTTAACGGCCGCGGTATTT	19	47.4
	F2a	GGCTGGAATGAAAGGTTTTACG	22	45.5
	F2b	GGCTGGAATGAAAGGTTTAACG	22	45.5
	F2c	GGCTGGTATGAAGGGTTTAACG	22	50
	F2d	CTGGAATGAAGGGTTTGACGA	21	47.6
	Reverse primer			
	R1a	CTGTAAATATGAACTCTGGAGAGGG	25	44
	R1b	CTGTAAATATGAACTCTGGAGGAGG	25	44
	R1c	TCGTAAATATGAACTCTGGAAGAGG	25	40
	R1d	CTGTAGATATGAACTCTGGAGGAAG	25	44
	R1e	CCTGTAAATTTGAACTCTGAAGGAAG	26	38.5
	R1f	TTCTTGTAAATTTGAACTCTGAAAGAAG	28	28.6
	R2a	CGAGGTCGCAAGCCTTT	17	58.8
	R2b	CGAGGTCGCAAGCCCT	16	68.8
	R2c	GAGGTCGCAAGCCCCT	16	68.8
	R2d	ACATCGAGGTCGCAATCTTTC	21	47.6
	R2e	CATCGAGGTCGCAAACTTTC	20	50
	R2f	ATCGAGGTCGCAATCCTTC	19	52.6
	R2g	CGAGGTCGCAAGCCTTC	17	64.7
	R2h	CGAGGTCGCAACCCTTC	17	64.7
	R2j	GAGGTCGCAAGCCCATC	17	64.7
	R2k	CGAGGTCGCAAGCCTC	16	68.8
	R2l	TCGAGGTCGCAAGCCC	16	68.8
	R2m	CGAGGTCGCAAGCCCTA	17	64.7
	R2n	AGGTCGCAAGCCCCAC	16	68.8
v2 combination 1	Forward primer			
	F1a	TTTAACGGCCGCGGTATAC	19	52.6
	F1b	TTTAACGGCCGCGGTATAT	19	47.4
	F1d	TTTAACGGCCGCGGTATTT	19	47.4
	F1ad02	TTTAACGGCCGCGGTATTC	19	52.6
	Reverse primer			
	R2e	CATCGAGGTCGCAAACTTTC	20	50
	R2f	ATCGAGGTCGCAATCCTTC	19	52.6
	R2g	CGAGGTCGCAAGCCTTC	17	64.7
	R2h	CGAGGTCGCAACCCTTC	17	64.7
	R2k	CGAGGTCGCAAGCCTC	16	68.8
	R2l	TCGAGGTCGCAAGCCC	16	68.8
	R2x1	CGAGGTCGCAAGCTCCACT	19	63.2
v2 combination 2	Forward primer			
	F1ad01	TTTAACGGCCGCGGTATCT	19	52.6
	F1ad03	TTTAACGGCCGCGGTACTC	19	57.9
	F1ad05	TTTAACGGCCGCGGTACAC	19	57.9
	F1ad06	TTTAACGGCCGCGGTAAAT	19	47.4
	F1ad07	TTTAACGGCCGCGGTACTT	19	52.6
	F1ad08	TTTAACGGCCGCGGTACAT	19	52.6
	F1ad09	TTTAACGGCCGCGGTAAAC	19	52.6
	F2x1	AATGAAGGGTTTAACGAATCATTAG	25	32
	F2x3	AATGAATGGTTTGACGAAATATCAG	25	32
	F2x4	AATGAATGGTTTGACGAAATATCGG	25	36
	F4x1	ACTTTTCAGTTAAAAGGCTGAAATTGT	27	29.6
	F4x2	ACTTTTCAGTTAAAAGGCTGAAATTTC	27	29.6
	F3x1	TTAACGAGGAATTGACTGTCTCATC	25	40
	F5x1(F3x2)	GGTGTTGACTGTCTCATTTAATTTGTG	27	37
	Reverse primer			
	R2a	CGAGGTCGCAAGCCTTT	17	58.8
	R2e	CATCGAGGTCGCAAACTTTC	20	50
	R2g	CGAGGTCGCAAGCCTTC	17	64.7
	R2k	CGAGGTCGCAAGCCTC	16	68.8
	R2l	TCGAGGTCGCAAGCCC	16	68.8
	R2x2	AACATCGAGGTCGCAACC	18	55.6
	R2x3	CAACATCGAGGTCGCAAAC	19	52.6

v3	Forward primer			
	v4_F1	GCAAAGGTAGCATAATCATTAGTCTTTTAATA	32	28
	v4_F2	GCAAAGGTAGCATAATCATTAGTCTCTTAATA	32	31
	v4_F3	GCAAAGGTAGCATAATCATTAGTCTTTTAAAA	32	28
	v4_F4	CAAAGGTAGCATAATCATTAGCCTTTTAATA	31	29
	v4_F5	GCAAAGGTAGCATGATCATTAGTCTTTTAATA	32	31
	v4_F6	GCAAAGGTAGCATAATCCTTAGTCTTTTAATA	32	31
	Reverse primer			
	v4_R1	AAGAAATTAAAGGTCGAACAGACCTATA	28	32
	v4_R2	AAGATTTTAAAGGTCGAACAGACCTATA	28	32
	v4_R3	AAGAATTTAAAGGTCGAACAGACCTATA	28	32
	v4_R4	AAGAGTTTAAAGGTTGAACAGACCTATA	28	32
	v4_R5	AAGAATATAAAGGTCGAACAGACCTATA	28	32
	v4_R6	GAGTTTAAAGGTCGAACAGACCTATA	26	38
	v4_R7	ATTTCAAAGGTCGAACAGACCTATA	25	36
	v4_R8	AAATTAAAGGTCGAACAGACCCATA	25	36
	v4_R9	AATTTAAAGGTCGAACAGACCCATA	25	36
	v4_R10	GGGTCGAACAGACCCATA	18	56
	v4_R11	TCATGTAAGAATTTAAAAGTCGAACAGACTTATA	34	26
	v4_R12	AAGAATTTAAAGGTCAAACAGACCTATT	28	29
	v4_R13	AAGAATTTAAAGGTTGAACAGACCTATT	28	29
	v4_R14	AGAATTTAAAGGTCGAACAGACCTATT	27	33
	v4_R15	AGATTTTAAAGGTCGAACAGACCTATT	27	33
	v4_R16	AGAATTTAAAGGTGGAACAGACCTATT	27	33
	v4_R17	AGAAATTAAAGGTCGAACAGACCTATT	27	33
	v4_R18	AGTTTAAAGGTCGAACAGACCTATT	25	36
	v4_R19	ATTCAAAGGTCGAACAGACCTATT	24	38
	v4_R20	TAAAGGTCGAACAGACCCATT	21	36
	v4_R21	GAATTTAAAGGTCGAACAGACCTATG	26	38
	v4_R22	GAATTTAAAGGTCGAACAGACCTATC	26	38
	v4_R23	AATTTAAAGGTCGAACAGACCTACA	25	36
	v4_R24	GGTCGAACAGACCCACA	17	59
	v4_R25	TTAAAGGTCGAACAGACCTAAACA	24	38

691

692 Table 4. Total number of reads obtained and the proportion of Odonata reads for each sample.

	v1		v2		v3	
ID	Read Num.	Prop. of Odonata	Read Num.	Prop. of Odonata	Read Num.	Prop. of Odonata
Paddy-1	47,159	100.0%	263,170	100.0%	40,448	97.6%
Paddy-2	427	0.0%	22,393	0.0%	1,354	0.0%
Paddy-3	27,439	99.9%	100,266	99.5%	64,880	74.2%
Paddy-4	4,885	0.1%	855	0.0%	2,490	0.0%
Paddy-5	354,671	75.9%	125,583	0.0%	160,145	0.0%
Paddy-6	28,110	98.5%	117,458	24.4%	63,820	18.8%
Paddy-7	21,181	98.4%	95,016	65.4%	55,632	42.8%
Paddy-8	481	0.0%	7	0.0%	327	49.8%
Paddy-9	77,837	0.0%	1,546	0.0%	58,050	0.0%
Paddy-10	5,569	0.0%	116,836	99.9%	64,867	49.3%
Pond-1	12	58.3%	243	2.5%	0	-
Pond-2	114,698	99.9%	57,470	93.3%	71	50.7%
Pond-3	23,433	99.5%	85,428	91.6%	28,807	89.7%
Pond-4	22,278	91.1%	2,865	84.7%	122,199	12.8%
Pond-5	250,969	99.7%	47,978	94.9%	18,454	18.3%
Pond-6	140,415	98.6%	34,297	99.3%	49,100	41.2%
Pond-7	233,312	98.4%	46,453	99.2%	61,839	20.7%
Pond-8	336,356	99.7%	46,066	97.6%	50,870	81.1%
Container-1	31,972	100.0%	103,636	95.3%	59,136	27.1%
Container-2	18	0.0%	13,598	0.0%	6,269	85.9%
Container-3	20,658	100.0%	15,829	98.6%	449	59.2%
Container-4	33,030	100.0%	91,506	100.0%	74,221	98.8%
Container-5	242,797	99.7%	51,872	100.0%	45,270	98.5%
Channel	14,236	99.2%	95,556	57.1%	86,563	71.6%
River-1	2,919	0.0%	65,259	0.0%	75,240	0.0%
River-2	11,871	0.0%	212,519	16.5%	67,137	0.0%
River-3	62,381	0.0%	143,240	0.0%	81,285	0.0%
River-4	16,569	0.0%	151,241	0.0%	117,303	0.0%
Negative controls	32,041	1.5%	694	19.7%	221	1.4%

693

Table 5. Species detected at each sampling site with 10 or more reads.

	Paddy 1–7			Paddy 8–10			Pond 1–5			Pond 6–8			Container 1–5			Channel			Stream		
	v1	v2	v3	v1	v2	v3	v1	v2	v3	v1	v2	v3	v1	v2	v3	v1	v2	v3	v1	v2	v3
Anax nigrofasciatus								+													
Anotogaster sieboldii																					
Ceriagrion melanurum													+	+	+						
Ceriagrion nipponicum												+		+	+						
Crocothemis servilia						+	+	+	+	+	+	+	+	+	+						
Davidius fujiama																				+	
Ischnura asiatica																					
Ischnura senegalensis										+											
Lanthus fujiacus		+	+														+	+			
Libellula angelina													+	+	+						
Lyriothemis pachygastra															+						
Mnais costalis	+	+	+													+	+	+			
Orthetrum albistylum							+	+	+	+	+	+	+								
Orthetrum japonicum																	+	+			
Orthetrum melania								+		+											
Paracercion calamorum										+											
Pseudothemis zonata							+	+	+				+								
Rhyothemis fuliginosa													+		+						
Stylogomphus suzukii																					
Sympecma paedisca OR *Indolestes peregrinus*																	+				
Sympetrum darwinianum	+	+	+		+	+	+						+			+	+	+			
Sympetrum depressiusculum OR *Sympetrum frequens*							+														
Species encountered during water sampling†	*S. darwinianum* (AN) (+)						*O. albistylum* (A) (+) *O. melania* (A) (+) *P. zonata* (AE) (+) *Sympetrum baccha* (E)			*C. nipponicum* (A) (+) *C. servilia* (A) (+) *O. albistylum* (A) (+) *O. melania* (A) (+) *R. fuliginosa* (A)			*A. nigrofasciatus* (AN) *C. melanurum* (AN) (+) *C. servilia* (AN) (+) *L. angelina* (AN) (+)			*S. darwinianum* (A) (+)			*Calopteryx cornelia* (A)		

†Species encountered during water sampling. Letters indicate the developmental stage of the individuals encountered: A, adult; N, nymph; E, exuviae. Plus signs (+) indicate species detected by eDNA.