Intra-individual polymorphisms in the mitochondrial COI gene of tick-killing *Ixodiphagus* wasps parasitizing *Haemaphysalis flava* ticks

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Abstract

Ixodid ticks are significant vectors of pathogens affecting both humans and animals. Biological control with natural enemies represents a sustainable tool for managing ticks. However, there is a substantial lack of knowledge about the natural enemies of ticks. Wasps of the genus *Ixodiphagus* (Encyrtidae) are currently the only known tick-specific parasitoids. While these wasps have been sporadically recorded worldwide, their presence in Eastern Asia is poorly documented. In this study, we investigated the occurrence of *Ixodiphagus* wasps in field-collected ticks reared on rabbits under laboratory conditions. Ticks were collected from the Hokkaido, Hokuriku, Chugoku, Shikoku, and Kyushu regions in Japan. Out of 1,933 *Haemaphysalis* ticks infested to rabbits, adult wasps emerged from 15 engorged ticks. All the ticks from which wasps emerged were morphologically and molecularly identified as *Haemaphysalis flava*. Additionally, wasp DNA was detected in unfed *H. flava* nymphs using a newly designed *Ixodiphagus*-specific PCR assay. Among nine experimental sites in the Chugoku region, *Ixodiphagus* wasps were detected at three sites, with parasitism rates ranging from 1.8% to 8.1%. Finally, the mitochondrial COI gene of four wasp and two tick samples were characterized using shotgun sequencing, direct sequencing, and in-fusion cloning approaches. Multiple intra-individual polymorphisms were observed in all the tested samples. Further studies are needed to investigate the relationship between *Ixodiphagus* wasps and Ixodid ticks. An increased understanding of these parasitoid wasps could contribute to future biological control measures against

ticks.

Keywords

Tick, *Ixodiphagus*, Encyrtidae, mitochondrial genome; biological control; parasitoid

1. Introduction

Encyrtid (Hymenoptera: Chalcidoidea) wasps are a species-rich family of arthropod parasitoids (Bistline-East et al., 2015; Burke and Sharanowski, 2024; Japoshvili et al., 2016). They play a crucial role in regulating host populations (Bokonon-Ganta and Neuenschwander, 1995; Kapranas and Tena, 2015). For this reason, Encyrtid wasps and other members of the Chalcidoidea are recognized as an important group as biological control agents against arthropod pests (Conti et al., 2021; Kapranas and Tena, 2015; Kenis et al., 2017).

Ixodid ticks are significant vectors of pathogens of humans and animals (Fang et al., 2023; Stanley et al., 2020; Talactac et al., 2018). Tick management is commonly performed with acaricidal chemicals, but their effectiveness is limited due to the increasing development of acaricide resistance (Dzemo et al., 2022). Alternatively, integrated pest management program, composed of multiple countermeasures such as chemicals, physical protection, and natural enemies, can be employed (Stafford et al., 2017). However, there is a substantial lack of knowledge about the natural enemies of ticks. Generalist predators – such as ants, beetles, pseudoscorpions, spiders, crabs, and mites – are known to eat ticks (Fei et al., 2023; Okabe et al., 2018; Samish et al., 2004; Samish and Alekseev, 2001; Showler et al., 2019; Van Veen et al., 2008; Vinogradov et al., 2024). Yet, such predators are often inefficient as biological control agents due to a lack of specificity. Currently, chalcid wasps, *Ixodiphagus* (Encyrtidae), are the only known tick-specific predators (Mwangi et al., 1991).

Female *Ixodiphagus* wasps lay their eggs inside the body of ticks by inserting their ovipositor through the host exoskeleton (Hu et al., 1998). These eggs then remain dormant until the tick feeds on a host. As with other parasitoids, the wasp larvae kill the tick host during development and later emerge as adult. To date, at least 16 species of *Ixodiphagus* wasps have been described and they are known to parasitize at least 15 species of Ixodid ticks across 6 genera (Hu et al., 1998; Ramos et al., 2023; Tachikawa, 1980). *Ixodiphagus* wasps have been recorded around the world including in Asia, Europe, Africa, North America, South America, and Oceania (Ramos et al., 2023). In Asia, these wasps have been recorded from South Asia, Southeast Asia, and Far Eastern Siberia, while there is no documented record from Eastern Asia aside from an incidental record from Japan (Tachikawa, 1980). The diversity of *Ixodiphagus* wasps is considered to be underestimated due to lack of sampling (Giannotta et al., 2024). Therefore, investigation in the Eastern Asia region is valuable for addressing this knowledge gap.

Molecular techniques have been useful for systematics and evolutionary studies of arthropods (Blaimer et al., 2023; Jasso-Martínez et al., 2022). These techniques are also applicable for species-specific detection and barcoding (Kurata et al., 2024). Mitochondrial genes are widely used for these purposes and are well-represented in genetic databases (Desalle et al., 2017; French et al., 2023). Several characteristics make mitochondrial genes useful for such studies: their high copy number within cells, conserved gene catalogues across species, and moderate mutation rates between species.

However, there are several difficulties in the use of mitochondrial genes for Hymenoptera characterization. Intra-individual variations on mitochondrial gene have been reported in a range of wasps and bees (Davies et al., 2022; Françoso et al., 2023; Magnacca and Brown, 2010; Ricardo et al., 2020; Yan et al., 2019). This phenomenon is caused by heteroplasmy, where individuals possess multiple sets of mitochondrial genes, and nuclear mitochondrial DNA segments (NUMTs), where fragments of mitochondrial DNA are inserted into the nuclear genome. Although one recent study presented the complete mitochondrial genomes of *Ixodiphagus* wasps (Giannotta et al., 2024), there has been no report of intra-individual variation of the mitochondrial genes of this important wasp genus. To collect reliable molecular data on *Ixodiphagus*, it is essential to precisely characterize these intra-individual polymorphisms.

In this study, we aimed to investigate the *Ixodiphagus* wasps in Japan to better understand their geographic distribution, and genetic characteristics.

2. Material and Methods

2.1. Ethical statement

All animal experiments were conducted in accordance with the guidance of Institute for Laboratory Animal Research, following the Fundamental Guidelines for Proper Conduct of Animal Experiment

and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The procedures were approved by the Animal Experiment Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Approval No. 22-0030). This study complies with the ARRIVE guidelines.

2.2. Detection of *Ixodiphagus* wasps from the field collected ticks by the tick infestation on the laboratory animals

Ixodiphagus wasps were detected from nymphal ticks collected from vegetation and wild animals. A total of 1,933 questing ticks were collected by flagging at 20 sampling sites across five regions in Japan between 2021 and 2024 (Figure 1, Supplementary Table 1 and Supplementary Table 2). Ticks were also collected from dead wild animals, including tanuki (*Nyctereutes viverrinus*) and badger (*Meles anakuma*). These ticks were unfed or partially fed nymphs. The collected ticks were morphologically identified based on previously described keys (Yamaguti et al., 1971). The tick species included in this study were *Haemaphysalis flava, Haemaphysalis formosensis, Haemaphysalis japonica, Haemaphysalis longicornis,* and *Haemaphysalis megaspinosa.* All the ticks were infested on the ears of female Japanese white rabbits (Slc: JW/CSK; Japan SLC, Shizuoka, Japan) using cotton bags to cover the left and right ears separately. Multiple species from the same sampling site or animal were infested in the same ear bag. The replete ticks were kept in Petri dishes under constant dark

126 conditions at 25℃ and 90% relative humidity until they molted or died due to drying, mold, or wasp
127 emergence. Wasp samples were kept in 80% ethanol at -20℃ upon their emergence from ticks until
128 being examined under a stereoscopic microscope and DNA extracted.
129 Emerged wasp samples used for molecular analysis were investigated morphologically by comparing
130 them with those reported previously (Erdős, 1955; Fiedler, 1953; Geevarghese, 1977; Giannotta et al.,
131 2024; Hayat and Kazmi, 2011; Hayat and Veenakumari, 2015; Heath and Cane, 2010; Nikol'skaya,
132 1950; Noyes, 2010; Quaraishi, 1958). Morphological characters were photographed with a digital
133 microscope VHX-7000 (KEYENCE Inc., Osaka, Japan) using a focus stacking function.
134
135 **2.3. Morphological and molecular identification of host ticks**
136 Tick carcasses ("mummies") after wasp emergence were subjected to morphological and molecular
137 species identification. Morphological identification was performed based on the previously described
138 keys (Yamaguti et al., 1971). Thereafter, the third and fourth legs on one side of the mummies were
139 cut for DNA extraction with HotSHOT protocol (Truett et al., 2000). The collected legs were placed
140 into 10 µl of 25 mM NaOH (pH12) and incubated for 30 minutes followed by neutralization with 10
141 µl of neutralizing buffer containing 100 mM Tris-HCl and 0.5 mM EDTA (pH8). PCR was performed
142 using a primer set (mt-rrs-1 and mt-rrs-2, Table 2) targeting the mitochondrial ribosomal RNA gene
143 with conditions indicated in Supplementary Table 3. PCR products were purified using the ExoSAP-

144 IT Express PCR Cleanup Reagent (Thermo Fisher Scientific, Tokyo, Japan) or the NucleoSpin® Gel
145 and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Sanger sequencing was performed with
146 the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using BigDye
147 Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The obtained sequences were compared
148 with a previously constructed sequence database (Takano et al., 2014).
149
150 **2.4. DNA extraction from wasps**
151 DNA was extracted from representative wasp samples using the NucleoSpin DNA Insect Kit
152 (Macherey-Nagel). Individual samples were cut at the head-thorax and thorax-abdomen joints to
153 extract DNA efficiently. The samples were incubated overnight at 55℃ in the mixture of 10 µl of
154 Proteinase K, 100 µl of Buffer BE, and 40 µl of Buffer MG, followed by extraction according to the
155 manufacturer's protocol. The exoskeletons after DNA extraction were kept in 80% ethanol at -20℃
156 for future investigation.
157
158 **2.5. Shotgun sequencing and data analysis**
159 Shotgun sequencing was performed with a representative wasp sample (sample code: WS03-03, Table
160 3). An Illumina sequencing library was prepared using the IDT for Illumina DNA/RNA UD Indexes
161 (Illumina, Hayward, CA, USA) in combination with the Illumina DNA Prep kit (Illumina). The library

was analyzed with the NextSeq 1000 sequence system using NextSeq™ 1000/2000 P1 XLEAP-SBS™ Reagent Kit (600 Cycles) (Illumina). Base-calling and trimming were performed with the Dynamic Read Analysis for GENomics (DRAGEN™) (Illumina). The FASTQ files were deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (https://www.ddbj.nig.ac.jp/index.html) under an accession number of PRJDB18816. The obtained reads were error-corrected and normalized by the BBNorm function, followed by mapping to a reference COI gene sequence of *Ixodiphagus hookeri* (JQ315225) using the Geneious Prime build 2022-11-28 (Kearse et al., 2012). Polymorphic sites were identified by the Geneious SNP/Variation function with a minimum variant frequency of 0.01.

2.6. Molecular detection of *Ixodiphagus* wasps in questing ticks

Molecular detection of *Ixodiphagus* wasps was conducted on the questing *H. flava* nymphs collected in Chugoku region (Figure 1, Supplementary Table 4). Two hundred and three ticks were collected by flagging vegetation at nine sampling sites in April and June of 2024. The samples were rinsed once with 70% ethanol then twice with molecular-grade water before homogenization using the Micro Smash MS-100R (TOMY, Tokyo, Japan) for two minutes at 2,500 rpm. DNA was extracted from the individual tick samples with DNAzol (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. The extracted DNA was used as a template for PCR with a newly designed primer set, 347F-H and 942R-H (Table 2 and Supplementary Table 3), which target a partial COI sequence of

Ixodiphagus. These primers were designed on the conserved regions between the observed sequence in the sample used for the shotgun sequencing (sample code: WS03-03) and the reference sequence of *I. hookeri* (JQ315225). Mixed bases were incorporated at the variable sites to account for the intra-individual polymorphisms.

2.7. Direct sequencing of COI PCR amplicons

A total of six samples were selected as a representative sample set for wasp COI gene sequence characterization (Table 3). The set included DNA from four adult wasps (sample codes: WS01-01, WS03-03, WS08-02, and WS09-01), collected from different sampling regions and tick hosts, and two questing ticks positive for wasps by PCR (sample codes: WN072 and WN203). The least damaged four wasps were chosen to facilitate future morphological investigation. DNA from each sample was used for PCR with the designed primer set as mentioned above (347F-H and 942R-H, Table 2) under the conditions detailed in Supplementary Table 3. The resulting PCR products were subjected to direct Sanger sequencing as described above. Electropherograms obtained were aligned using the software Geneious and manually examined to detect any ambiguities with multiple peaks within a 551-bp region between primer binding sites.

2.8. Cloning and sequencing of COI gene fragments

198 Cloning of the COI gene fragments was performed on the same set of DNA as direct sequencing
199 analysis. Insert COI gene fragments were generated using a high-fidelity PCR enzyme and modified
200 primer set (In-Fusion_347F-H and In-Fusion_942R-H, Table 2), targeting the same region as the direct
201 sequencing, as indicated in Supplementary Table 3. The fragments were ligated with the PCR-
202 linearized pUC19 vector (Takara Bio Inc., Shiga, Japan) using the In-Fusion HD Cloning Kit (Takara
203 Bio Inc.). Competent *Escherichia coli* DH5α cells (Nippon Gene, Tokyo, Japan) were transformed
204 with the ligation products and pre-incubated at 37℃ for an hour. The transformed cells were
205 subsequently inoculated onto LB agar plates containing ampicillin and X-gal, and incubated overnight
206 at 37℃. Individual colonies were selected to amplify the insert sequences using the EmeraldAmp
207 MAX PCR Master MIX (Takara Bio Inc.) with a primer set (M13 Pfrimer M4 and M13 Primer RV,
208 Table 2). A total of 10 clones from each sample were sequenced by Sanger sequencing as mentioned
209 above. The obtained clone sequences were aligned and trimmed using Geneious software. The
210 sequences were translated with invertebrate mitochondrial genetic code. Putative NUMTs were
211 determined based on two criteria: (1) the presence of insertion or deletion causing a frameshift, (2) the
212 presence of substitution resulting in a stop codon in the coding region. Clones determined as putative
213 NUMTs were excluded from further analysis. The remaining nucleotide sequences were re-aligned
214 and subsequently used to construct a median-joining haplotype network using the PopART v1.7
215 (Bandelt et al., 1999; Leigh and Bryant, 2015). The obtained sequences were deposited in the DDBJ

under the accession numbers LC849322 – LC843959 (Supplementary Table 5).

3. Results

3.1. Detection of wasps in engorged ticks

Out of the 1,933 *Haemaphysalis* nymphs experimentally infested on rabbits, adult wasps were obtained from a total of 15 engorged ticks collected from four regions (Figure 1, Table 1). All tick species parasitized by the wasps were identified as *H. flava* both molecularly and morphologically. Among the questing ticks infested on rabbits, 2% (1/48 from Shikoku and 7/340 from Hokuriku) of the ticks were found to be parasitized by the wasps. Four wasps emerged from a single tick in Shikoku, while an average of seven wasps emerged from seven ticks in Hokuriku (Table 1). Among the ticks collected from wild animals and subsequently infested on rabbits, 8% (4/36) and 3.5% (4/114) of the ticks collected from badgers and tanukis, respectively, were found to be parasitized by the wasps. The mean number of wasps that emerged from a single tick was 10 from badgers and five from tanuki (Table 1).

3.2. Morphological characterization of the wasps

The four samples (sample codes: females - WS01-01, WS03-03, and WS09-01; male - WS08-02, Table

3) used for sequencing analysis were morphologically examined (Figure 2, Supplementary Figures 1, 2 and 3).

For females, the 16 described species of *Ixodiphagus* wasps can be broadly classified based on the characteristics of the torulus and the scape of the antennae. In our samples (sample codes: WS01-01, WS03-03, and WS09-01), the torulus were above the ventral margin of compound eyes, and the scape were relatively wide and short (Figures 2A and 2B, Supplementary Figures 1A, 1B, and 3A). These characteristics correspond to the eight species of *Ixodiphagus*, including *I. hookeri, Ixodiphagus sagarensis, Ixodiphagus tadystes, Ixodiphagus brunneus, Ixodiphagus mysorensis, Ixodiphagus godens, Ixodiphagus taiaroensis,* and *Ixodiphagus theilerae*. However, our samples were different from those eight species by the following characteristics; (i) the axillae were hardly raised and lacked distinct posterior ridges; (ii) the fore wings were relatively large, one to three lines of the setae were below the submarginal vein proximally, the basal cell had a naked area, and the linea calva closed with one to two rows of setae posteriorly (Figures 1D, 1E and 1F, Supplementary Figures 1D, 1F, 3B, and 3D). In *I. taiaroensis* and *I. theilerae*, the fore wings are small (brachypterous). In *I. godens*, the axillae are clearly raised with distinct posterior ridges. In the fore wings of *I. hookeri, I. sagarensis, I. tadystes, I. mysorensis,* and *I. brunneus*, the basal cell mostly covered with setae, and the linea calva are open.

For males, the information on morphological characteristics is available for eight species of *Ixodiphagus*, including *I. hookeri, I. sagarensis, I. mysorensis, I. taiaroensis, I. theilerae, Ixodiphagus*

texanus, Ixodiphagus hirtus, and *Ixodiphagus biroi*. Our sample (sample code: WS08-02) was different from those eight species based on the morphological features of axillae and fore wings as observed in females (Supplementary Figure 2). Therefore, the samples obtained from this study could not be identified to the species level and were considered as putative novel species.

3.3. Detection of wasp DNA in the questing ticks

The parasitism rates of wasps were molecularly assessed among questing *H. flava* nymphs collected at nine experimental sites in Chugoku. Three sites tested positive for wasp parasitism, with rates of 8.1% (3/37), 1.8% (1/57), and 5.7% (2/35) (Supplementary Table 4).

3.4. Detection of polymorphisms on mitochondrial COI gene by shotgun sequencing

A total of 4,025,830 raw reads were obtained from a wasp sample (sample code: WS03-03) using the NextSeq platform. After trimming, error-correction, and normalizing of the raw reads, a total of 593,736 reads were kept for the downstream analysis. By mapping the reads to a reference COI gene sequence of *I. hookeri* (JQ315225), putative COI sequence of the sample WS03-03 was constructed using 1,969 reads with a mean coverage of × 143.4. In the 979-bp region of the putative COI gene, 38 intra-individual polymorphic sites including 23 transitions and 15 transversions were observed (Figure

3).

3.5. Verification of intra-individual polymorphisms by sanger sequencing

The intra-individual polymorphisms in the COI gene were verified through two Sanger sequencing approaches, direct sequencing of COI PCR amplicons and sequencing of the inserted COI fragments by in-fusion cloning. For this purpose, a set of six representative DNA samples (four wasps and two ticks) were analyzed (Table 3). The direct sequencing of COI PCR amplicons yielded double peaks in the electropherograms of all six samples, with a total of 17 double-peak sites observed across the amplified region (Figure 4). For the in-fusion cloning sequencing, ten different clones were randomly selected per sample and sequenced to generate a final data set of 60 sequences of 474 bp in length. The sequence alignment identified a total of 38 different haplotypes from six samples (Figure 5, Supplementary Table 5). The most frequent haplotype, MH1, was found in all samples and was presumed to be a putative wild type for the subsequent analysis. Four haplotypes (MH35, MH36, MH37, and MH38) and one haplotype (HM20) had a 10-bp insertion or a single nucleotide deletion, respectively, causing frameshift and premature termination, while two haplotypes (MH18 and MH28) had a nucleotide substitution causing nonsense mutation (Figure 5). These seven haplotypes were considered putative NUMTs and were excluded from the subsequent analysis. Among the remaining 31 haplotypes, a total of 46 single nucleotide polymorphic sites (SNPs) were observed, including 24

transitions, 20 transversions, and two multiple variations (Supplementary Table 6). Of those SNPs, 16 were synonymous, while 30 led to non-synonymous amino-acid changes compared to the wild type (Figure 5).

The three sequencing methods - shotgun sequencing, direct sequencing, and cloning - were compared based on the SNPs within the 474-bp overlapping region of the COI gene of a wasp sample (sample code: WS03-03, Figure 6). Shotgun sequencing detected the highest number of polymorphic sites (16 sites) followed by direct sequencing (12 sites) and cloning (nine sites). Two polymorphic sites were identified as shared among all three methods, whereas 12 sites were shared between shotgun sequencing and direct sequencing methods. Four polymorphic sites were unique to shotgun sequencing, and seven sites were unique to cloning.

3.6. Haplotype network analysis of wasp COI gene

A haplotype network was constructed based on 474-bp partial COI gene sequences, consisting of 31 haplotypes obtained from six representative samples (Table 3) and one reference sequence of *I. hookeri* from France (JQ315225). The haplotypes did not form any distinct clustering based on the sequence origin (Supplementary Figure 4). The presumed wild type, MH1, along with four other haplotypes, MH2, MH3, MH4, and MH5, were closely linked and positioned as central haplotypes in the network. Other haplotypes radiated with one to 11 nucleotide differences from these central haplotypes. None

306 of the haplotypes were identical to the reference *I. hookeri* haplotype.

307

308

309 **4. Discussion**

310 This study presents the first documented record of *Ixodiphagus* wasps in Japan since 1980 (Tachikawa,
311 1980). This is also the first confirmed record of the wasp parasitizing *H. flava.* The representative
312 specimens showed significant morphological differences from the previously recorded species, *I.*
313 *sagarensis,* in Japan. This observation indicates that these *Ixodiphagus* wasps may belong to a different
314 species. The detection of *Ixodiphagus* wasps across three geographically distinct regions – Hokuriku,
315 Chugoku and Shikoku – indicates a broad distribution of these parasitoid wasps in Japan. Also, given
316 the wide distribution of *H. flava* throughout East Asia (St John et al., 2021; Yamaguti et al., 1971;
317 Zhang et al., 2019), it is likely that the *Ixodiphagus* wasps detected in this study may also occur outside
318 of Japan as well. Since *H. flava* is a significant vector with public health implications (Fang et al.,
319 2023; Fournier et al., 2002; Yoshii et al., 2017), the presence of these parasitic wasps may represent a
320 valuable tool for the control of ticks and tick-borne diseases in the region. Further taxonomic studies
321 are needed to classify these wasps.

322 *Ixodiphagus* and other Encyrtidae wasps are known to parasitize multiple species (Bistline-East et al.,
323 2015; Bowman, 1979; Geden and Moon, 2009; Larrousse et al., 1928). This suggests that the species

detected from *H. flava* might also parasitize other tick species. However, in this study, wasps emerged solely from *H. flava*, despite the inclusion of four other *Haemaphysalis* species in the infestation experiments using rabbits (Supplementary Table 1). This specificity may indicate that wasp development was successful in only *H. flava*. *Haemaphysalis flava* has a broad host range including mammals and birds (Yamaguti et al., 1971; Yamauchi, 2001). Therefore, it is possible that this species might have allowed wasps to develop properly even with unusual blood sources, such as laboratory rabbits in this study. This could be tested by performing *Ixodiphagus*-specific PCRs on other field-collected *Haemaphysalis* species to detect *Ixodiphagus* DNA. Another hypothesis is that the detected wasps were host-specific to *H. flava*. In a trial conducted in Kenya, a reduction in *Amblyomma variegatum* infestations was observed, while the number of *Rhipicephalus appendiculatus* remained unchanged after releasing *Ixodiphagus* wasps (Mwangi et al., 1997). This outcome underscores the potential of *Ixodiphagus* wasps in species-specific tick management. Investigation of the host range of *Ixodiphagus* wasp species will support future applications in biological control.

Intra-individual polymorphisms in the mitochondrial COI gene were observed in all tested wasps. There was no association of the observed COI haplotypes with geographic origins of the wasps or animal species from which the tick hosts were collected (Table 3, Supplementary Figure 4). This suggests that NUMTs and/or heteroplasmic mitochondrial genomes are common across populations and generations of the *Ixodiphagus* wasps. While NUMTs and heteroplasmic mitochondrial genomes

342 are relatively common in other Hymenoptera species (Calvignac et al., 2011; Davies et al., 2022;
343 Françoso et al., 2023; Magnacca and Brown, 2010; Ricardo et al., 2020), mechanisms underlying these
344 genetic features remain poorly understood. Inconsistencies in the polymorphic sites were observed
345 among three sequencing methods (Figure 6). This could be caused by several factors. Shotgun
346 sequencing was likely the least biased, but the continuities between short reads were not detectable.
347 In direct sequencing, minor mutations may be masked due to manual observation of double peaks in
348 electropherograms. In cloning sequencing, the observation of haplotypes could have been biased due
349 to harmful insertion sequences for competent cells which could be poorly detected after cloning. To
350 obtain genuine mitochondrial sequences, other methods such as long-read sequencing, cDNA
351 sequencing, mtDNA purification during DNA extraction, or limiting dilution could be employed
352 (Calvignac et al., 2011). Careful approaches are needed in the molecular investigation of *Ixodiphagus*
353 wasps.
354 The present study has several technical limitations. The infestation experiments using rabbits may
355 have underestimated the infestation rate of wasps in *H. flava*. Questing ticks were collected mostly
356 from vegetation by flagging and then infested on rabbits. Since *Ixodiphagus* wasps are known to locate
357 feeding ticks by following host vertebrate odor plumes (López-López et al., 2024; Philip, 1931), the
358 ticks tested may not have had sufficient exposure to the wasps. In addition, the emergence rate might
359 be lower than the parasitism rate, as wasps have previously been observed to fail to emerge inside

ticks, as found in dissections (Dominguez et al., 2023). In the molecular detection of wasps from questing ticks, several factors may have influenced the results. The amount of wasp DNA in the questing ticks could be low due to its dormant state as wasp eggs (Hu and Hyland, 1998), reducing detection sensitivity. Additionally, the seasonality of wasps may have affected both the infestation and molecular detection experiments (Larrousse et al., 1928). Further studies are needed to investigate the relationship between *Ixodiphagus* wasps and Ixodid ticks.

The distribution, host range, and molecular information presented in this study are just a small fragment of the true diversity of *Ixodiphagus*. Information on the *Ixodiphagus* wasps have mostly been gained through incidental observations. There are only a few documentations to catalog *Ixodiphagus* species in certain regions (Noyes, 2010). Integrated approaches with morphological and molecular techniques are needed to classify this group with active sampling from host ticks. Furthermore, to apply these wasps for biological tick management in the future, investigations into host range and general biological factors under laboratory conditions are necessary. Comprehensive approaches including these investigations will contribute to elucidating the diversity of *Ixodiphagus* wasps.

5. Conclusion

This study provides insights into the distribution and mitochondrial genetic diversity of *Ixodiphagus* wasps in Japan, with implications for their broad distribution in Eastern Asia. We successfully detected *Ixodiphagus* wasps in nymphal *H. flava* ticks, highlighting their potential role in natural tick population control. Through molecular methods, including shotgun sequencing, direct sequencing, and in-fusion cloning, we characterized intra-individual polymorphisms in the wasp's mitochondrial COI gene, leading to the detection of potential NUMTs and/or heteroplasmic mitochondrial genomes. Further studies are needed to investigate the relationship between *Ixodiphagus* wasps and Ixodid ticks.

Acknowledgements

We would like to thank Yoshimitsu Higashiura from the Yamaguchi Agricultural Technology Department for his invaluable insights and advice on the taxonomy of Encyrtid wasps. We are grateful to Aiko Onuma and Yukari Horio for their technical support with sequencing and valuable advice. We also appreciate the members of the Laboratory of Parasitology, Faculty of Veterinary Medicine, Hokkaido University for their helpful discussions and support.

Author contributions

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Funding sources

This study was funded by Japan Society for the Promotion of Science (JSPS) KAKENHI (JP20KK0151, JP23K23770, JP23K14090 and JP24KK0133); the Japan Agency for Medical Research and Development (AMED), Japan (JP23wm0225034); Research Fellowships of JSPS for Young Scientists; the World-leading Innovative and Smart Education (WISE) Program (1801) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; and Hokkaido University DX Doctoral Fellowship, Japan.

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Figure 1: Sampling regions of the ticks used for experimental infestation on rabbits.

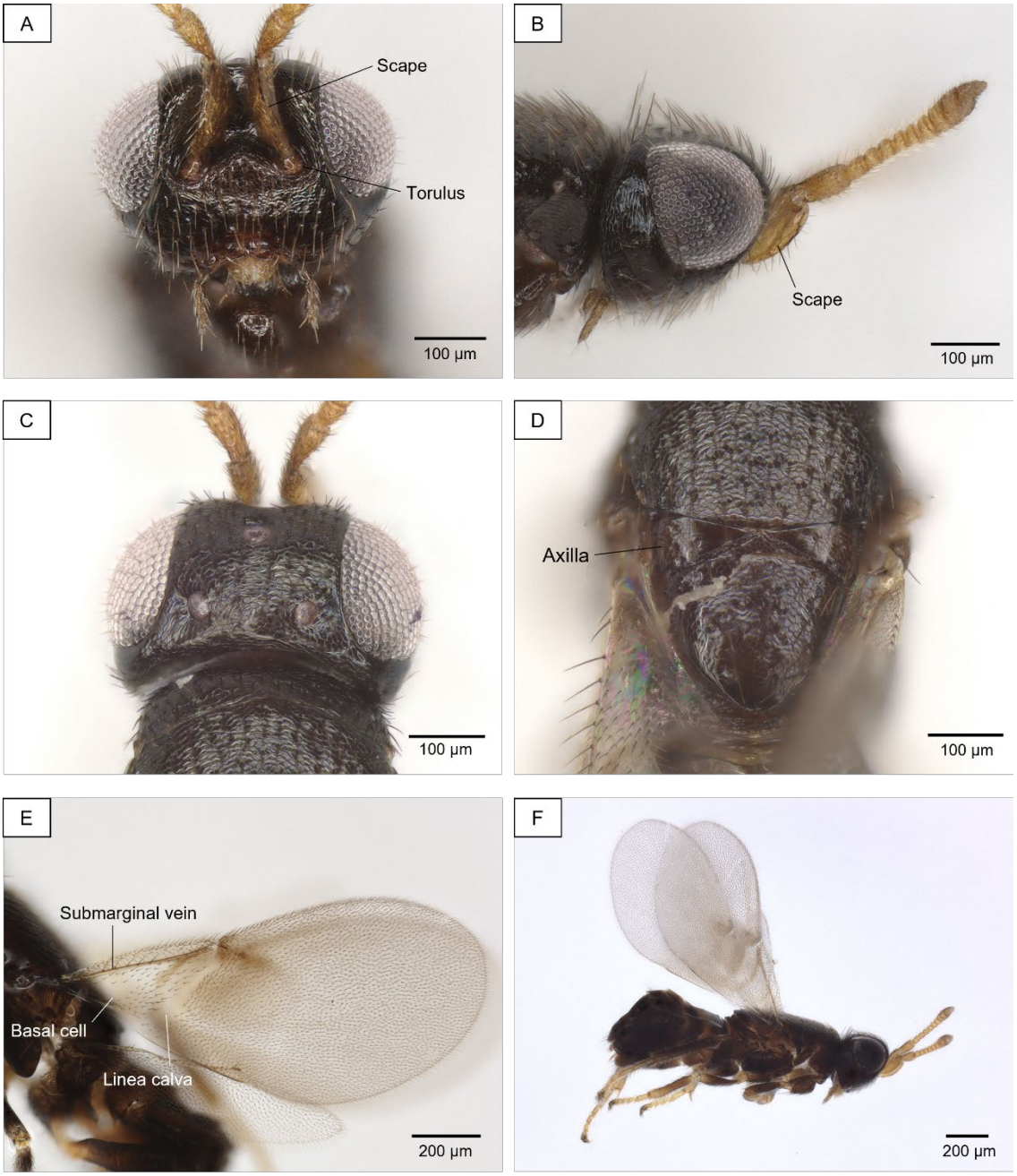


Figure 2: An *Ixodiphagus* wasp emerged from *Haemaphysalis flava* (WS03-03).

Head A) frontal, B) lateral, and C) dorsal views. D) Mesosoma dorsal view. E) Fore wing. F) Habitus.

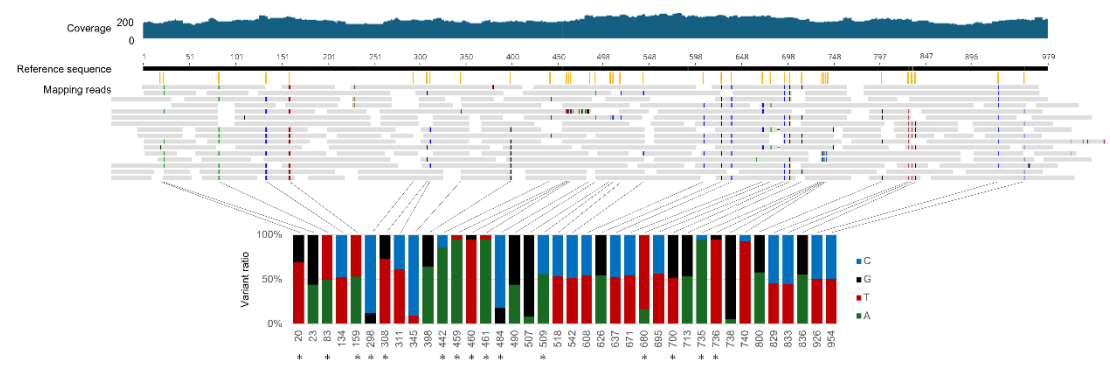


Figure 3: Polymorphisms in the partial COI gene detected by mapping of shotgun sequencing reads against a reference sequence of *Ixodiphagus hookeri*.

The Illumina sequence reads obtained from a wasp (sample code: WS03-03) were mapped against a reference *Ixodiphagus hookeri* COI gene (JQ315225). The upper panel shows the coverage of mapping reads. The middle panel shows the reference sequence and the mapped reads. Yellow bars indicate polymorphic sites. Disagreements to the consensus sequence are highlighted by representative colors in the mapped reads. The lower panel shows the ratio of observed variants at polymorphism sites. The numbers below represent the polymorphic site regions corresponding to the reference sequence. Asterisks represent transversions.

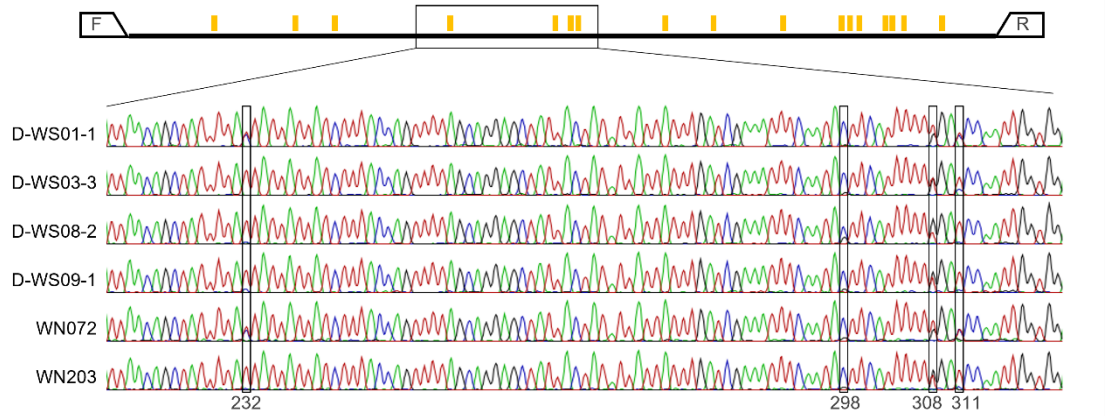


Figure 4: Electropherograms obtained by the direct sequencing of *Ixodiphagus* COI PCR amplicons.

The upper panel indicates the position of double-peak sites (yellow bars) in partial COI PCR amplicons. Trapezoids indicate primer binding regions (F, 347F-H; R, 942R-H). The alignment on the lower panel indicates ambiguities on the electropherograms. The numbers below represent the corresponding positions to the *Ixodiphagus hookeri* partial COI sequence (JQ315225).

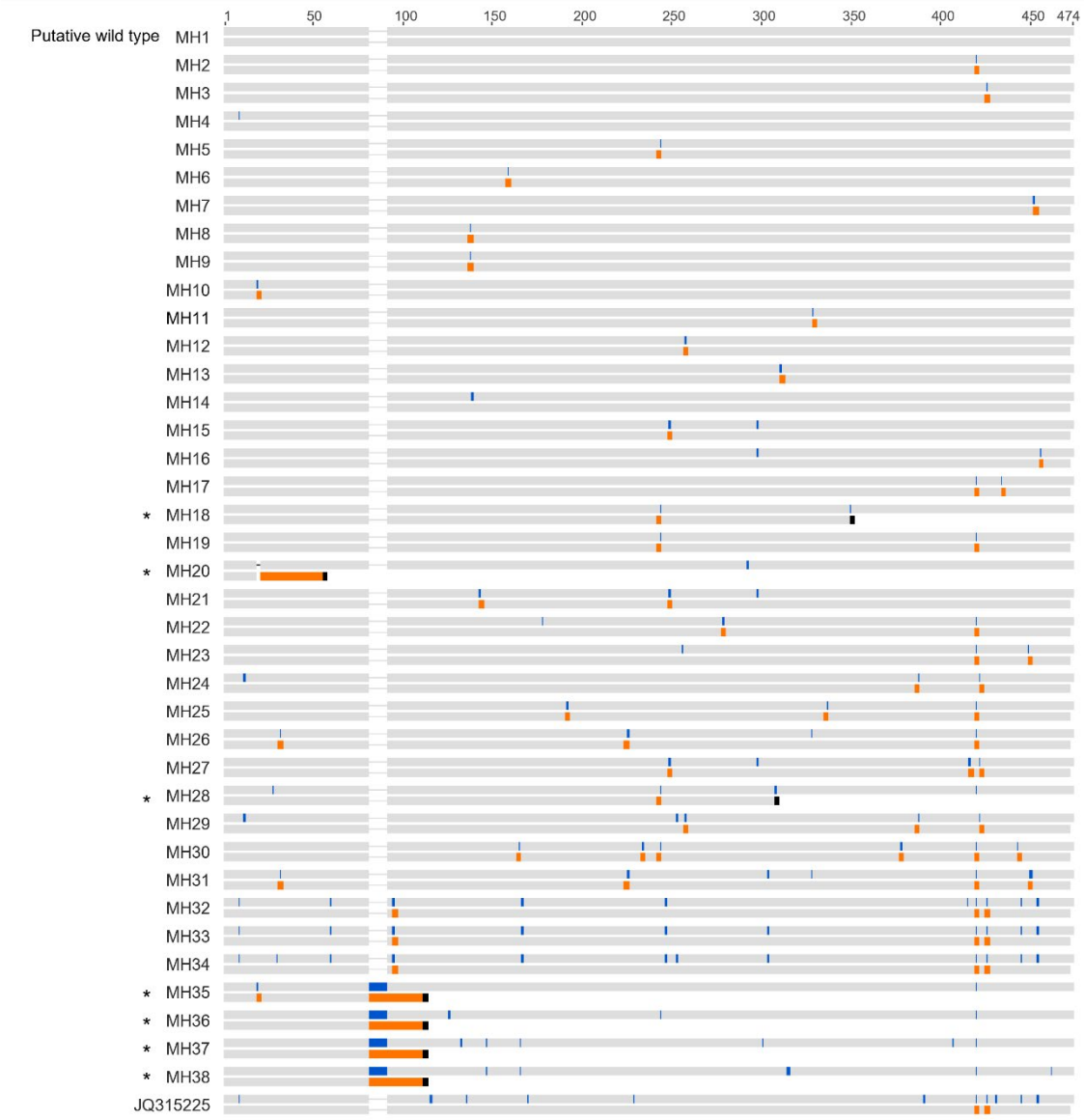


Figure 5: Alignment of the 38 COI haplotype sequences obtained in the present study.

For each haplotype, the nucleotide sequence is shown in the first row, and the translated amino-acid sequence is shown in the second row. Nucleotide and amino-acid differences from the putative wild-type (MH1) sequence are highlighted in blue and orange, respectively. Stop codons are indicated in black. Asterisks denote putative NUMTs.

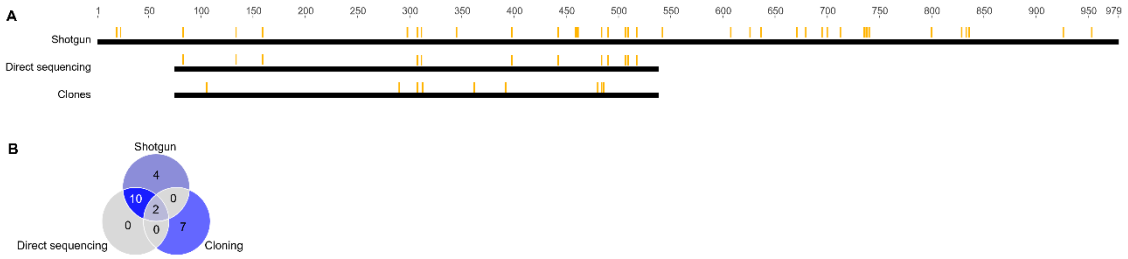


Figure 6: Comparison of intra-individual polymorphisms in the partial COI gene of a wasp sample (WS03-03) using three different sequencing approaches.

A) Regions of SNPs detected by each method. B) Number of common SNPs in the preserved 474 bp region across the three methods.

Table 1: Summary of *Ixodiphagus* wasps emerged from the ticks experimentally infested on rabbits.

Infestation ID*[1]	Region	Tick source	Number of ticks infested	Number of ticks parasitized by wasps (%)	Mean number of wasps per single tick (Number of wasps per each tick)
1	Shikoku	Vegetation	48	1 (2.1)	4 (4)
7	Hokuriku	Vegetation	340	7 (2.1)	7 (ND*[2])
14	Chugoku	Badger	36	3 (8.3)	10 (20, 5, 5)
15	Chugoku	Tanuki	114	4 (3.5)	5 (2, 4, 5, 7)

*[1]Infestation ID corresponds to the IID in Supplementary Table 1 including the details of the experiment.

*[2]Number of wasps per each tick were not counted due to the emergence from multiple ticks in the same enclosure.

Table 2: Primers and amplification protocols performed in this study.

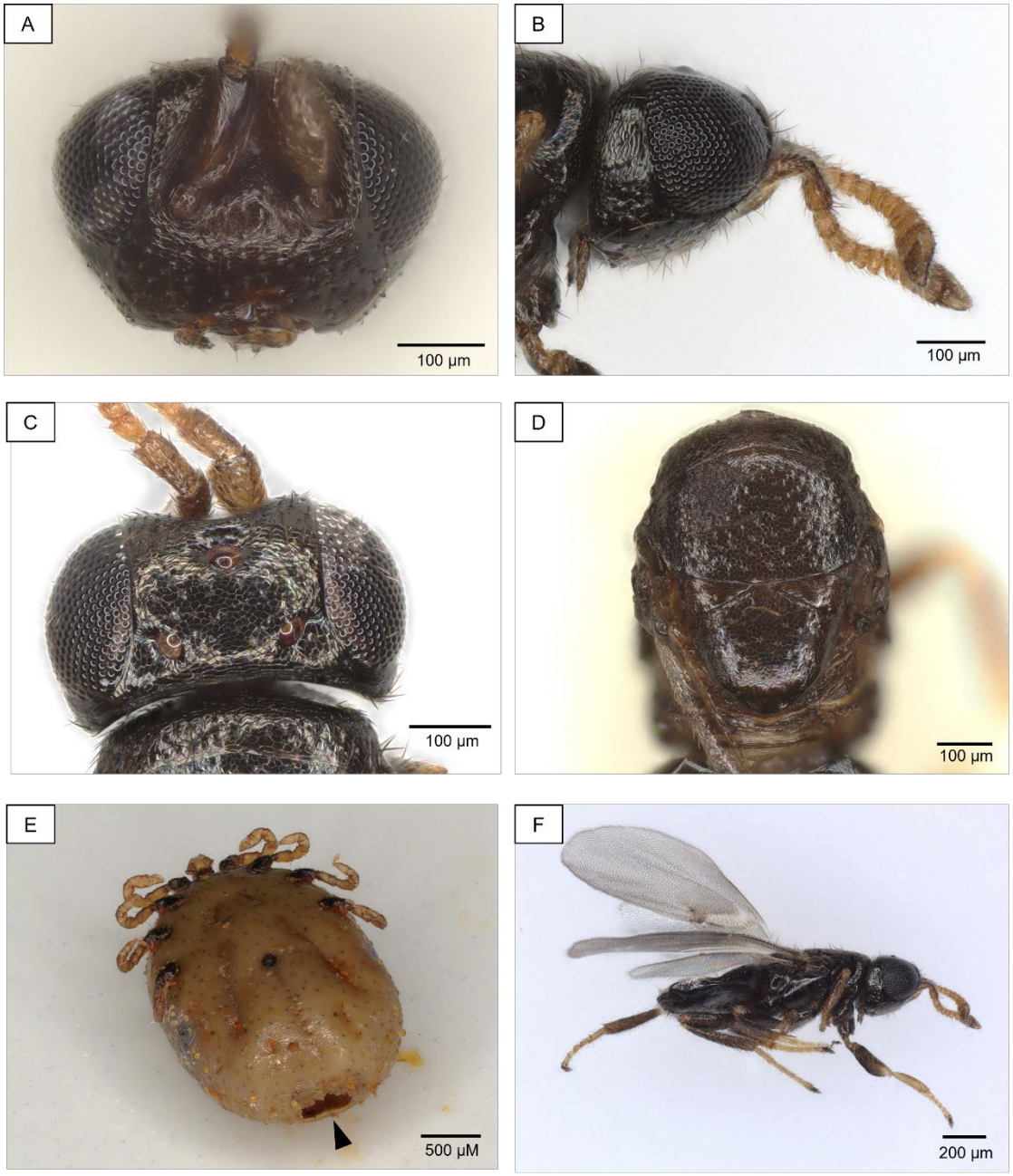
Primer name	Target organism (Target gene)	Purpose	Primer sequence (5 → 3)	Reference
mt-rrs-1	Ixodid tick	Tick species identification	CTGCTCAATGATTTTTTAAATTGCTGTGG	(Ushijima et al., 2003)
mt-rrs-2	(16S rRNA)		CCGGTCTGAACTCAGATCAAGTA	
347F-H	*Ixodiphagus* sp.	Detection and direct sequencing	TTGATTCAGGAACKGGRACTGGA	This study
942R-H	(COI)		TACTCCTGTTGGTACAGCAATA	
In-Fusion_347F-H	*Ixodiphagus* sp.	Insert sequence preparation for in-fusion cloning	AAAACGACGGCCAGTTTGATTCAGGGACTGGGACTG	This study
In-Fusion_942R-H	(COI)		GACCATGATTACGCCTACTCCTGTTGGTACAGC	
M13 Primer M4	pUC19 vector	Amplifying insertion sequencing	GTTTTCCCAGTCACGAC	Takara Bio Inc.
M13 Primer RV	(COI)		CAGGAAACAGCTATGAC	

Table 3: Information of the representative samples used for the verification of intra-individual polymorphisms in COI gene.

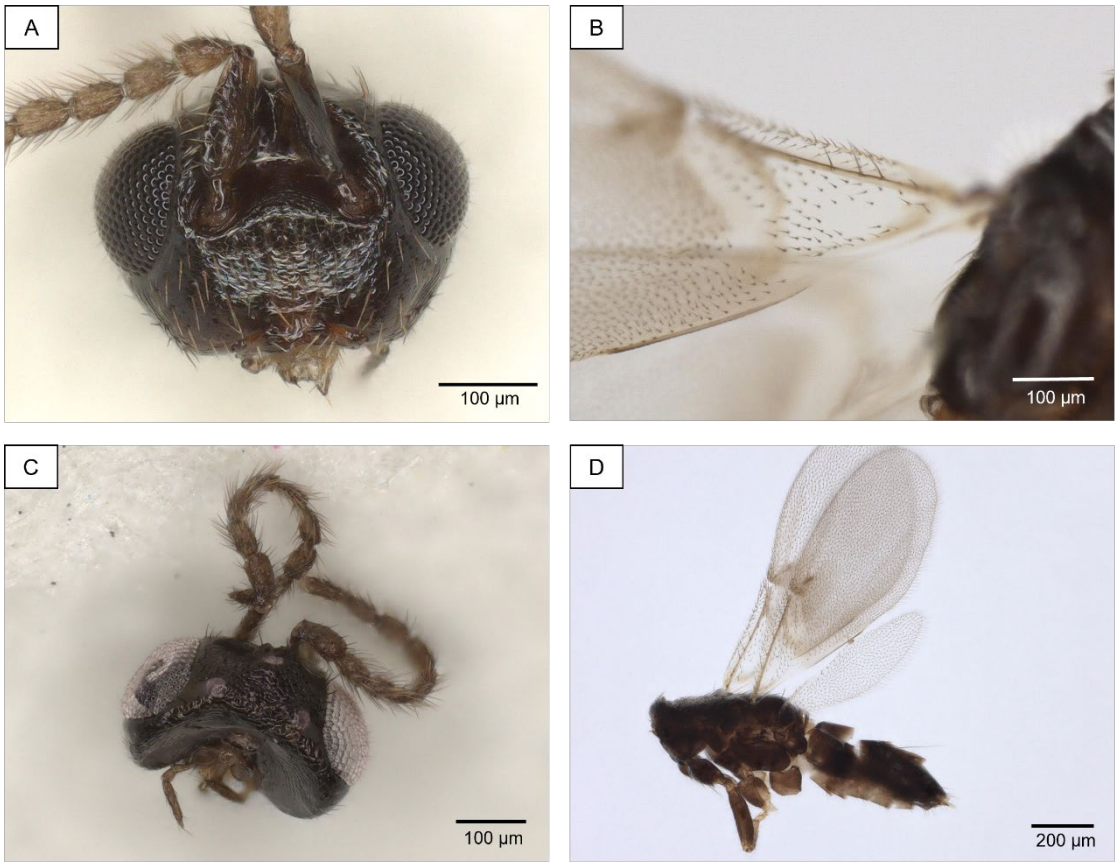
Sample code	Infestation ID*[1]	Prefecture	Tick source	Sex
WS01-01	1	Kochi	Vegetation	F
WS03-03	7	Ishikawa	Vegetation	F
WS08-02	14	Shimane	Tanuki	M
WS09-01	15	Shimane	Badger	F
WN072	NA*[2]	Shimane	Vegetation	NA*[2]
WN203	NA*[2]	Shimane	Vegetation	NA*[2]

*[1] Infestation ID corresponds to the IID in Supplementary Table 1 including the details of the experiment.

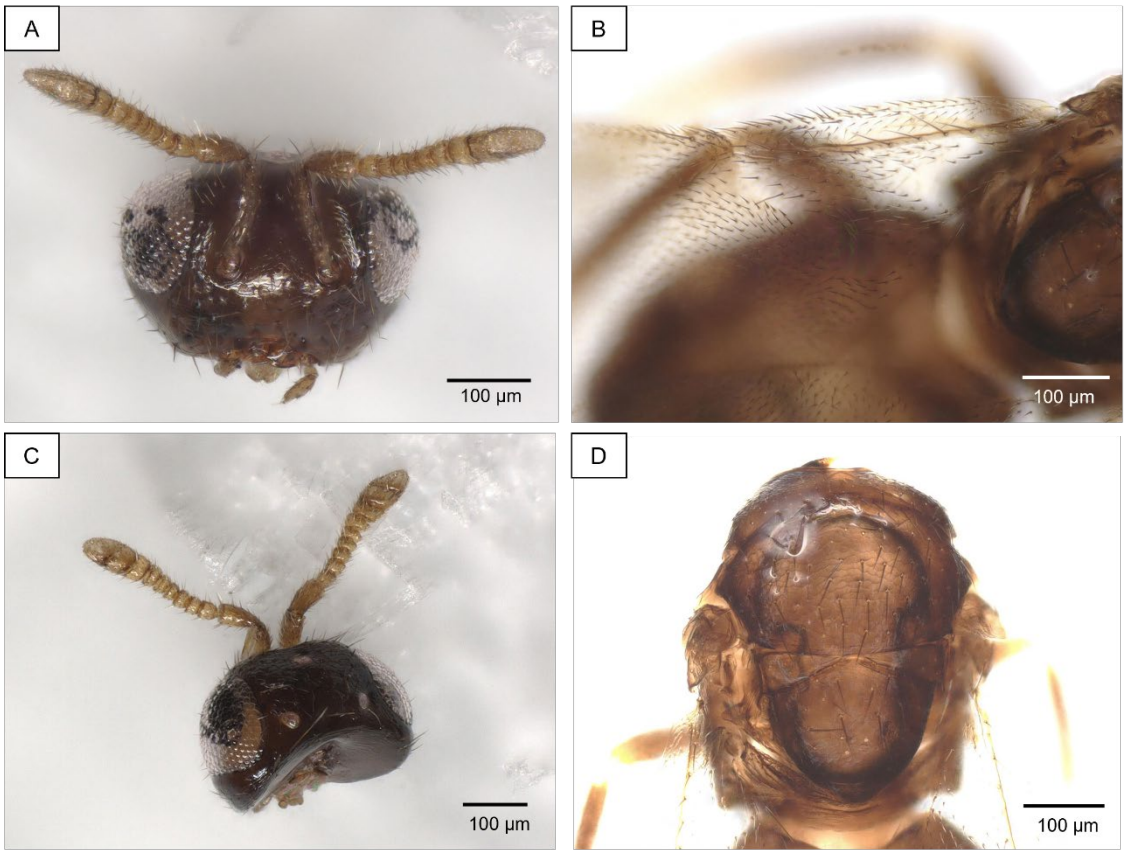
*[2] NA, not applicable.



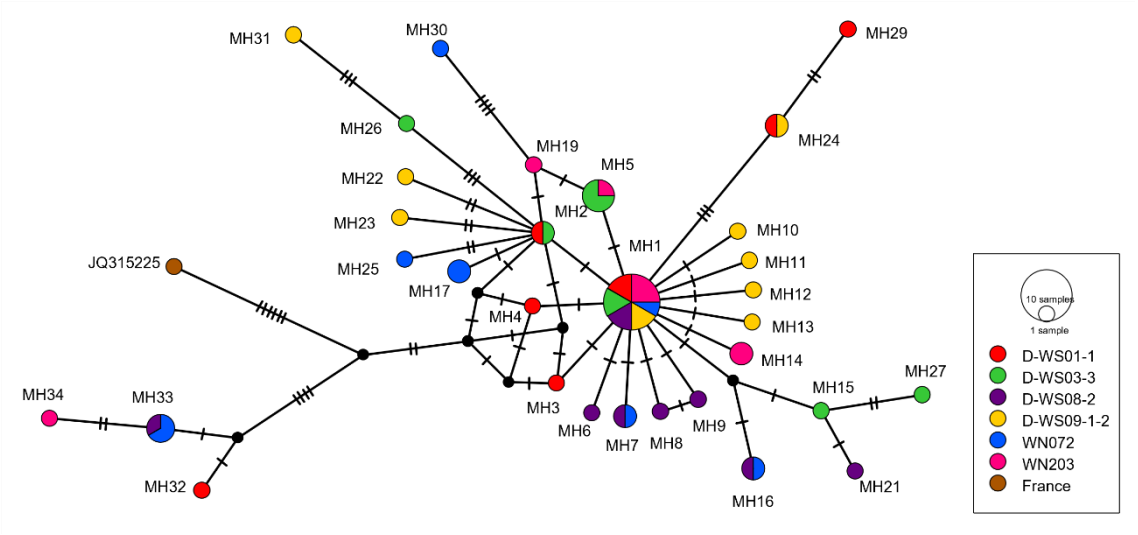
Supplementary Figure 1: An *Ixodiphagus* wasp (WS01-01) emerged from *Haemaphysalis flava*. Head in A) frontal, B) lateral, and C) dorsal views. D) Mesosoma in dorsal view. E) Mummy. The arrowhead indicates the aperture due to the wasp emergence. F) Habitus.



Supplementary Figure 2: An *Ixodiphagus* wasp (WS08-02) emerged from *Haemaphysalis flava*. Head in A) frontal and C) dorsal views. B) Fore wing. D) Habitus without the head.



Supplementary Figure 3: An *Ixodiphagus* wasp (WS09-01) emerged from *Haemaphysalis flava*. Head in A) front and C) dorsal views. B) Fore wing. D) Mesosoma in dorsal view.



Supplementary Figure 4: Median-joining haplotype network of the *Ixodiphagus* wasp partial COI gene sequences.

A haplotype network was constructed based on 474-bp partial COI gene sequences, consisting of 31 haplotypes obtained from six representative samples (Table 3) and one reference sequence of *I. hookeri* from France (JQ315225). Circle represents unique haplotypes, size of circle corresponds to the frequency of its haplotype, color represents wasp or tick sample, and lines on the branches correspond to number of mutations between haplotypes.

Supplementary Table 1: Details of the experimental tick infestations on rabbits.

IID[*1]	Tick species[*2]					Region	Prefecture	GPS	Sampling year	Sampling month	Tick source	Number of infested nymphs	Number of ticks parasitized by wasps (%)
	Hfla	Hfor	Hjap	Hlon	Hmeg								
1	+	+			+	Shikoku	Kochi	33.46, 133.33	2021	Dec.	Vegetation	48	1 (2.1)
2				+		Kyushu	Fukuoka	33.37, 130.80	2022	Mar.	Vegetation	120	0
3	+	+		+		Kyushu	Fukuoka	33.29, 130.60	2022	Mar.	Vegetation	140	0
4	+	+				Kyushu	Fukuoka	33.50, 130.30	2022	Mar.	Vegetation	80	0
5			+		+	Hokkaido	Hokkaido	43.38, 144.14	2022	Sep.	Vegetation	7	0
6	+		+	+	+	Hokkaido	Hokkaido	42.11, 143.00	2022	Sep.	Vegetation	145	0
7	+			+		Hokuriku	Ishikawa	37.49, 137.18	2023	Mar.	Vegetation	340	7 (2.1)
8			+		+	Hokkaido	Hokkaido	42.11, 143.00	2023	May	Vegetation	180	0
9					+	Hokkaido	Hokkaido	42.44, 142.48	2023	Oct.	Vegetation	160	0
10	+					Shikoku	Kochi	33.47, 133.33	2023	Dec.	Vegetation	100	0
11	+					Shikoku	Kochi	33.44, 133.30	2023	Dec.	Vegetation	103	0
12	+	+				Shikoku	Kochi	33.48, 133.36	2023	Dec.	Vegetation	120	0
13					+	Hokkaido	Hokkaido	42.11, 143.00	2024	Apr.	Vegetation	240	0
14	+	+				Chugoku	Shimane	35.58, 133.13	2024	Mar.	Badger[*3]	36	3 (8.3)
15	+			+		Chugoku	Shimane	35.52, 133.04	2024	Mar.	Tanuki[*3]	114	4 (3.5)
										Total		**1,933**	**15**

[*1] IID (Infestation ID) corresponds to individual tick infestations conducted on a single ear of each rabbit. Each infestation was performed on separate ears.

[*2] Infested ticks were identified morphologically but not counted individually. Abbreviations for tick species: Hfla, *Haemaphysalis flava*; Hfor, *Haemaphysalis formosensis*; Hhys, *Haemaphysalis hystricis*; Hlon, *Haemaphysalis longicornis*; Hmeg, *Haemaphysalis megaspinosa.*

[*3] Ticks collected from animals were infested on laboratory rabbits to allow them to fully engorge.

Supplementary Table 2: Summary of the ticks experimentally infested on rabbits.

		Number of ticks infested	Number of ticks parasitized by wasps (%)
Region	**Hokkaido**	732	0
	Hokuriku	340	7 (2.1)
	Chugoku	150	7 (4.7)
	Shikoku	371	1 (0.27)
	Kyushu	340	0
Tick source	**Vegetation**	1,783	8 (0.45)
	Badger	36	3 (8.3)
	Tanuki	114	2 (3.5)

Supplementary Table 3: PCR protocols employed in the present study.

Primer name	PCR kit	Cycling conditions
mt-rrs-1 **mt-rrs-2**	Tks Gflex (Takara Bio Inc.)	94℃ for 1 min and 45 cycles of 98℃ for 10 sec, 60℃ for 15 sec, and 68℃ for 30 sec
347F-H **942R-H**	KOD-Plus-Neo (TOYOBO)	94℃ for 2 min and 40 cycles of 98℃ for 10 sec, 60℃ for 30 sec, and 68℃ for 30 sec
In-Fusion_347F-H **In-Fusion_942R-H**	Takara Ex Premier (Takara Bio Inc.)	94℃ for 1 min and 40 cycles of 98℃ for 10 sec, 55℃ for 15 sec, and 68℃ for 30 sec
M13 Primer M4 **M13 Primer RV**	EmeraldAmp MAX PCR Master MIX (Takara Bio Inc.)	30 cycles of 98℃ for 10 sec, 55℃ for 30 sec, and 72℃ for 1.5 min

Supplementary Table 4: Sampling site information and results of molecular detection of wasps among questing nymphal *Haemaphysalis flava*.

Site ID	Sampling year	Sampling month	GPS	Number of examined ticks	Number of PCR positive ticks (%)
S03	2024	Apr.	35.37, 133.52	37	3 (8.1)
S04	2024	Apr.	35.34, 133.59	8	0
S05	2024	Apr.	35.29, 133.56	18	0
S06	2024	Apr.	35.15, 133.28	38	0
IZM02	2024	Jun.	35.42, 132.69	1	0
IZM05	2024	Jun.	35.43, 132.80	1	0
IZM08	2024	Jun.	35.24, 132.67	35	2 (5.7)
IZM09	2024	Jun.	35.29, 132.79	57	1 (1.8)
IZM10	2024	Jun.	35.38, 132.89	8	0
Total				203	6 (3.0)

Supplementary Table 5: Observed haplotypes of COI gene by in-fusion cloning.

Mitochondrial haplotype	Sample code						Nucleotide polymorphism			Amino-acid polymorphism			Accession ID
	WS01-01	WS03-03	WS08-02	WS09-01	WN072	WN203	Deletion	Insertion	Substitution	Missense	Nonsense	Frameshift	
MH1	2	2	2	2	1	3							LC843922
MH2	1	1	0	0	0	0	-	-	+	+	-	-	LC843923
MH3	1	0	0	0	0	0	-	-	+	+	-	-	LC843924
MH4	1	0	0	0	0	0	-	-	+	-	-	-	LC843925
MH5	0	3	0	0	0	1	-	-	+	+	-	-	LC843926
MH6	0	0	1	0	0	0	-	-	+	+	-	-	LC843927
MH7	0	0	1	0	1	0	-	-	+	+	-	-	LC843928
MH8	0	0	1	0	0	0	-	-	+	+	-	-	LC843929
MH9	0	0	1	0	0	0	-	-	+	+	-	-	LC843930
MH10	0	0	0	1	0	0	-	-	+	+	-	-	LC843931
MH11	0	0	0	1	0	0	-	-	+	+	-	-	LC843932
MH12	0	0	0	1	0	0	-	-	+	+	-	-	LC843933
MH13	0	0	0	1	0	0	-	-	+	+	-	-	LC843934
MH14	0	0	0	0	0	2	-	-	+	-	-	-	LC843935
MH15	0	1	0	0	0	0	-	-	+	+	-	-	LC843936
MH16	0	0	1	0	1	0	-	-	+	+	-	-	LC843937
MH17	0	0	0	0	2	0	-	-	+	+	-	-	LC843938
MH18	0	0	0	0	1	0	-	-	+	+	+	-	LC843953
MH19	0	0	0	0	0	1	-	-	+	+	-	-	LC843939
MH20	0	1	0	0	0	0	+	-	+	+	+	+	LC843954

MH21	0	0	1	0	0	0	-	-	+	+	-	-	LC843940
MH22	0	0	0	1	0	0	-	-	+	+	-	-	LC843941
MH23	0	0	0	1	0	0	-	-	+	+	-	-	LC843942
MH24	1	0	0	1	0	0	-	-	+	+	-	-	LC843943
MH25	0	0	0	0	1	0	-	-	+	+	-	-	LC843944
MH26	0	1	0	0	0	0	-	-	+	+	-	-	LC843945
MH27	0	1	0	0	0	0	-	-	+	+	-	-	LC843946
MH28	0	0	1	0	0	0	-	-	+	+	+	-	LC843955
MH29	1	0	0	0	0	0	-	-	+	+	-	-	LC843947
MH30	0	0	0	0	1	0	-	-	+	+	-	-	LC843948
MH31	0	0	0	1	0	0	-	-	+	+	-	-	LC843949
MH32	1	0	0	0	0	0	-	-	+	+	-	-	LC843950
MH33	0	0	1	0	2	0	-	-	+	+	-	-	LC843951
MH34	0	0	0	0	0	1	-	-	+	+	-	-	LC843952
MH35	0	0	0	0	0	1	-	+	+	+	+	+	LC843956
MH36	1	0	0	0	0	0	-	+	+	+	+	+	LC843957
MH37	1	0	0	0	0	0	-	+	+	+	+	+	LC843958
MH38	0	0	0	0	0	1	-	+	+	+	+	+	LC843959

Supplementary Table 6: Observed polymorphisms of nucleotides and amino-acids in COI genes by in-fusion cloning.

Position	Nucleotide polymorphism* (Wild Type/ Variant 1 / Variant 2)	Polymorphism type	Variant frequency	Amino-acid polymorphism
9	T/A	Transversion	27/4	Synonymous
12	T/C	Transition	29/2	Synonymous
19	A/T	Transversion	30/1	Non-synonymous
30	A/G	Transition	30/1	Synonymous
32	A/G	Transition	29/2	Non-synonymous
60	T/C	Transition	28/3	Synonymous
85	A/T	Transversion	28/3	Non-synonymous
128	C/A/T	Transversion/Transition	29/1/1	Non-synonymous
129	T/C	Transition	30/1	Synonymous
133	G/A	Transition	30/1	Non-synonymous
149	C/A	Transversion	30/1	Non-synonymous
155	T/C	Transition	30/1	Non-synonymous
157	T/C	Transition	28/3	Synonymous
168	T/C	Transition	30/1	Synonymous
182	C/A	Transversion	30/1	Non-synonymous
216	T/A	Transversion	29/2	Non-synonymous
224	C/G	Transversion	30/1	Non-synonymous
234	T/G	Transversion	28/3	Non-synonymous
237	T/C	Transition	28/3	Synonymous
239	C/G	Transversion	28/3	Non-synonymous
243	T/C	Transition	29/2	Synonymous
246	T/A	Transversion	30/1	Synonymous
248	G/A	Transition	29/2	Non-synonymous
269	A/T	Transversion	30/1	Non-synonymous
288	T/C	Transition	27/4	Synonymous
294	T/C	Transition	28/3	Synonymous
301	G/A	Transition	30/1	Non-synonymous
318	T/C	Transition	29/2	Synonymous
319	C/A	Transversion	30/1	Non-synonymous
327	T/A	Transversion	30/1	Non-synonymous
368	A/C	Transversion	30/1	Non-synonymous

378	T/A	Transversion	29/2	Non-synonymous
405	A/G	Transition	30/1	Synonymous
406	T/A	Transversion	30/1	Non-synonymous
410	G/C	Transversion	19/12	Non-synonymous
412	A/T/G	Transversion/Transition	28/2/1	Non-synonymous
416	A/G	Transition	27/4	Non-synonymous
424	G/A	Transition	30/1	Non-synonymous
433	G/A	Transition	30/1	Non-synonymous
435	C/A	Transversion	28/3	Synonymous
439	G/T	Transversion	30/1	Non-synonymous
440	C/T	Transition	30/1	Non-synonymous
441	T/G	Transversion	30/1	Non-synonymous
442	C/T	Transition	30/1	Non-synonymous
444	C/T	Transition	28/3	Synonymous
446	A/G	Transition	30/1	Non-synonymous

*Polymorphic sites detected only in putative NUMTs were excluded.