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Morphological and Genetic Analyses Suggest Some Strains under *Tiphodytes gerriphagus* (Hymenoptera: Platygasteridae: Scelioninae), an Egg Parasitoid of Water Striders

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Abstract We conducted morphological observation and DNA analysis of Japanese *Tiphodytes*. In morphological observation, not only was *T. gerriphagus* identified, but also five strains were confirmed from individuals of *Tiphodytes* identified and morphological characters of the five strains except for *T. gerriphagus* did not match previous described *Tiphodytes* species. In ML tree based on 28S and COI sequence data analyzed from individuals of *Tiphodytes* identified, *T. gerriphagus* and the five strains were formed and the nodes of this species and the strains were well supported by bootstrap. The *Fst* values of 28S and COI sequence data analyzed from *Tiphodytes* collected indicated genetic differentiation between pairs of *T. gerriphagus* and each strain, and between pairs of strains. These results indicate that the genus *Tiphodytes* in Japan consists of at least *T. gerriphagus* and five strains, which probably include different species.

Introduction

Ecological research into several groups of species has been severely disrupted owing to a lack of adequate taxonomic knowledge. An inconvenient result is an evident mismatch between the amount of ecological science and the functional importance of these species groups (Griffith, 2012; Griffiths & Santos, 2012; Halme *et al.*, 2015). Difficulties with identification may result from natural phenotypic variation within a species, the involvement of morphologically cryptic taxa, limitations of morphological keys, the need to identify partial specimens that lack important taxonomic characteristics, and the shortage of experienced taxonomists required for identification (Hebert *et al.*, 2003). A combination of morphological identification and DNA techniques including DNA barcoding is being developed to overcome such problems (Floyd *et al.*, 2009; Moritz & Cicero, 2004).

Parasitoid wasps in the genus *Tiphodytes* (Hymenoptera: Platygasteridae) are well-known egg parasitoids of water striders (Hemiptera: Gerridae) around the world (Mineo *et al.*, 2009). Sixteen species of the genus *Tiphodytes* have been described globally (Mineo *et al.*, 2009, 2010; Rajmohana,

2012). Two species of *Tiphodytes*, *T. gerriphagus* (Marchal, 1900) and *T. godavari* Masner, 1972, have been recorded from the Palearctic region (Marchal, 1900; Masner, 1972; Mineo *et al.*, 2009). In Japan *T. gerriphagus* has been reported (Hirayama & Kasuya, 2009, 2010, 2015; Ito, 1980), however, *T. godavari* has not. *Tiphodytes gerriphagus* is important for ecological and evolutionary research, because adult females of this parasitoid wasp have been seen diving and actively swimming in water (Mineo *et al.*, 2010). Species identification in the genus *Tiphodytes* is based solely on morphological observation (Masner, 1972; Mineo *et al.*, 2009, 2010). However, it is sometimes difficult to identify parasitoid wasps because of overlapping morphological variation among intra- and inter- species (Bouček & Askew, 1968; Miller, 1970; Yoshimoto, 1983; Bernardo *et al.*, 2007). Therefore, we need further tool for identifying species of *Tiphodytes*.

In the present study, we found several different strains of *Tiphodytes* from Japan by using observation of morphological characters and DNA analysis techniques.

Materials and methods

Collection and preservation of *Tiphodytes* for morphological observation and molecular analysis

The samples of *Tiphodytes* used for morphological observation and DNA analysis were collected in yellow pan traps (YPT) (diameter 15 cm × height 4 cm) (Solo, Virginia, USA) (Yamagishi, 2004) with detergent placed in Okayama and Aichi. A series of YPT were placed for one hour at waterside of ponds and rivers (Tables 1–2). Parasitoid wasps collected were stored in 70% ethanol for morphological observation and in 100% ethanol for DNA analysis. Voucher specimens will be deposited in Western Region Agricultural Research Center and Meijo University.

Morphological observation and terminology

For microscopic study, ethanol-stored specimens were



Fig. 1. Female habitus of *Tiphodytes gerriphagus* (Marchal).

Table. 1. Collection data of *Tiphodytes* used in morphological and genetic analysis.

Sample no.	Collection date	Collection locality		Accession no.	
				28S	COI
<i>T. gerriphagus</i> -1 (♀)	4 July 2011	Nagoya, Aichi, Japan	35°10' 8" N, 137°1' 14" E	AB908396	AB909463
<i>T. gerriphagus</i> -2 (♀)	4 July 2011	Nagoya, Aichi, Japan	35°10' 8" N, 137°1' 14" E	AB908397	AB909464
<i>T. gerriphagus</i> -3 (♀)	4 July 2011	Nagoya, Aichi, Japan	35°10' 8" N, 137°1' 14" E	AB908398	AB909465
<i>T. gerriphagus</i> -4 (♀)	4 July 2011	Nagoya, Aichi, Japan	35°10' 8" N, 137°1' 14" E	AB908399	AB909466
<i>T. gerriphagus</i> -5 (♀)	4 July 2011	Nagoya, Aichi, Japan	35°10' 8" N, 137°1' 14" E	AB908401	AB909468
Strain 1-1 (♀)	6 July 2011	Nisshin, Aichi, Japan	35°7' 53" N, 137°1' 15" E	AB908403	AB909472
Strain 1-2 (♀)	6 July 2011	Nisshin, Aichi, Japan	35°7' 53" N, 137°1' 15" E	AB908404	AB909473
Strain 1-3 (♀)	6 July 2011	Nisshin, Aichi, Japan	35°7' 53" N, 137°1' 15" E	AB908405	AB909474
Strain 1-4 (♀)	6 July 2011	Nisshin, Aichi, Japan	35°7' 53" N, 137°1' 15" E	AB908406	AB909475
Strain 2-1 (♀)	6 May 2011	Nisshin, Aichi, Japan	35°7' 53" N, 137°1' 15" E	AB908407	AB909478
Strain 2-2 (♀)	6 May 2011	Nisshin, Aichi, Japan	35°7' 53" N, 137°1' 15" E	AB908408	AB909479
Strain 3-1 (♀)	27 Jun 2012	Ibara, Okayama, Japan	34°36' 22" N, 133°24' 53" E	AB908411	AB909483
Strain 3-2 (♀)	27 Jun 2012	Ibara, Okayama, Japan	34°36' 22" N, 133°24' 53" E	AB908412	AB909484
Strain 3-3 (♀)	27 Jun 2012	Ibara, Okayama, Japan	34°36' 22" N, 133°24' 53" E	AB908413	AB909485
Strain 3-4 (♀)	27 Jun 2012	Ibara, Okayama, Japan	34°36' 22" N, 133°24' 53" E	AB908414	AB909486
Strain 3-5 (♀)	27 Jun 2012	Ibara, Okayama, Japan	34°36' 22" N, 133°24' 53" E	AB908415	AB909487
Strain 4-1 (♀)	10 July 2012	Ibara, Okayama, Japan	34°36' 22" N, 133°24' 53" E	AB908421	AB909490
Strain 4-2 (♀)	10 July 2012	Ibara, Okayama, Japan	34°36' 22" N, 133°24' 53" E	AB908422	AB909491
Strain 5-1 (♀)	10 July 2012	Ibara, Okayama, Japan	34°36' 22" N, 133°24' 53" E	AB908424	AB909492
<i>Trimorus</i> sp.*	—	—	—	DQ888498	DQ888424
<i>Tnteleia</i> sp.*	—	—	—	DQ888502	DQ888427

*: These sequences reported by Murphy *et al.* (2007)Table. 2. Number of *Tiphodytes* specimens collected in YPT during 2011–2012.

<i>Tiphodytes</i> spp.	Collection locality (habitat)				
	Toyota, Aichi (pond)	Nagoya, Aichi (pond)	Shitara, Aichi (river)	Nisshin, Aichi (river)	Ibara, Okayama (river)
<i>T. gerriphagus</i>	10♀, 4♂	14♀, 6♂	—	—	—
Strain 1	29♀, 47♂	—	—	5♀, 4♂	—
Strain 2	—	—	12♀, 10♂	3♀	—
Strain 3	—	—	8♀, 22♂	1♀	46♀, 4♂
Strain 4	—	—	19♀, 3♂	—	2♀
Strain 5	—	—	3♂	—	3♀, 1♂

— Species or strains of wasps were not collected

dried by the method described in Lu *et al.* (2012). In the present study, the morphological terminology followed that of Masner (1980). Morphological characters of head sculpture, female antenna, shape of fore wing, venation, and shape and striation of metasoma were used for comparison. Dry specimens were identified as the genus *Tiphodytes* by having body relatively slender, smooth and glossy; skaphion of mesoscutum well developed; metasoma distinctly elongate; tergites 1 and 2 longitudinally costate, following tergites smooth; tergite 1 at most as long as wide or shorter; female antenna with 7 segments between scape and clava (Masner, 1972). Authentic specimens of *T. gerriphagus* identified by Dr. Masner in 1996 and deposited in the Entomological Laboratory, Meijo University, Nagoya, Japan, were compared with the collected specimens. *Tiphodytes gerriphagus* can be distinguished from the described *Tiphodytes* species by having body length 1.0–1.1 mm; legs, including coxae, black or blackish brown; wings clear; fore wing as long as body length; stigmal vein long; postmarginal vein absent; notauli represented by shallow depressions; femora not swollen; T1 (tergite 1) slightly broader than length; T2 with central striae reaching anterior 4/5 of T2 and lateral striae gradually shortened; T3 smooth. We compared the following morphological characters between the dry specimens and known *Tiphodytes* species: body length, occipital carina, central keel of frons, female clava, relative length or shape of fore wing, color of wings, postmarginal vein, relative length of marginal ciliae to width of fore wing, shape of notauli,

relative length of T1 to mesoscutum, aspect ratio of T1 and striae of T2. Dry specimens were observed under a binocular microscope (Olympus SZX, Tokyo, Japan).

DNA extraction and PCR amplification

We used partial gene regions of nuclear genes 28S (28S) and the mitochondrial DNA region encompassing partial sequences of cytochrome oxidase subunit I (COI). DNA was extracted nondestructively from a single adult female in 20 µL of a sterile 5% solution of Chelex-100 (Bio-Rad, Tokyo, Japan) in ultrapure water. Ultrapure water sterilized by autoclave was incubated with 1.4 µL of proteinase K (20 mg/mL) for at least 72 h at 56 °C. The specimens were boiled for 3 min at 99.9 °C to inactivate the proteinase K and were then used as templates for PCR. PCR amplification of 28S were conducted with 16 µL of AmpliTaq Gold™ 360 Master Mix (PE Applied Biosystems, Tokyo, Japan), 1.3 µL of forward and reverse primers (10 pmol/µL), 1 µL DNA template, and 13.4 µL of sterile water in a total PCR reaction volume of 33 µL. PCR amplification of COI were conducted with 10 µL of Ampdirect® Plus (Shimadzu Corporation, Kyoto, Japan), 0.1 µL of BioTaq™ HS DNA Polymerase (Nippon Genetics Co. Tokyo, Japan), 1 µL of forward and reverse primers (10 pmol/µL), 0.5 µL DNA template, and 7.4 µL of sterile water in a total PCR reaction volume of 20 µL. All reactions were done in an ABI Thermal Cycler (GeneAmp® PCR System 9700; PE Applied Biosystems, Tokyo, Japan) with the following program: an initial denaturing step at 96 °C for 10 min; 40

Table 3. Pairwise *Fst* values pairs of *T. gerriphagus* and each strain, or between pairs of strains in 28S sequences.

<i>Tiphodytes</i> spp.	<i>T. gerriphagus</i>	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
<i>T. gerriphagus</i>	-					
Strain 1	1**	-				
Strain 2	1*	1	-			
Strain 3	1***	1**	1*	-		
Strain 4	1*	1	1	1*	-	
Strain 5	1*	1	1	1*	1	-

P* < 0.05%, *P* < 0.01% and ****P* < 0.001%Table 4. Pairwise *Fst* values pairs of *T. gerriphagus* and each strain, or between pairs of strains in *COI* sequences.

<i>Tiphodytes</i> spp.	<i>T. gerriphagus</i>	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
<i>T. gerriphagus</i>	-					
Strain 1	0.8989***	-				
Strain 2	0.9539**	1**	-			
Strain 3	0.9654***	0.9961***	0.9951**	-		
Strain 4	0.8995*	0.9898*	0.9925	0.9915*	-	
Strain 5	0.9061	1	1	0.9934	0.9636	-

P* < 0.05%, *P* < 0.01% and ****P* < 0.001%

cycles of 95 °C for 1 min; an annealing step for 1 min at the temperatures described below; 72 °C for 1 min 30 s; and a final extension step of 72 °C for 7 min. Primers 28Sa (5'–GAC CCG TCT TGA AAC ACG GA–3') and 28Sb (5'–TCG GAA GGA ACC AGC TAC TA–3') (Whiting *et al.*, 1997) were used with an annealing temperature of 50 °C to amplify the region of 28S. Primers LCO1490 (5'–GGT CAA CAA ATC ATA AAG ATA TTG G–3') and HCO2198 (5'–TAA ACT TCA GGG TGA CCA AAA AAT CA–3') (Folmer *et al.*, 1994) were used with an annealing temperature of 45 °C to amplify the region of *COI*. Successful amplification was determined by electrophoresing 5 µL of the PCR mixture on a 1% agarose gel, staining with ethidium bromide, and observing under a UV transilluminator.

Sequencing and analysis

PCR products were sequenced by direct sequencing as described by Hagimori *et al.* (2006). The PCR products were purified by ultrafiltration using a Microcon ® YM-100 (Merck Millipore Corporation, Darmstadt, Germany). A dye-labeled terminator cycle-sequencing reaction were conducted with a BigDye® DNA Sequencing Kit, version 3.1 (PE Applied Biosystems, Tokyo, Japan). The reaction products were analyzed with an ABI PRISM 3130xl Genetic Analyzer (PE Applied Biosystems, Tokyo, Japan). The two primer sets used were the same as those used for PCR. DNA of each sample were sequenced twice for both strands. We sequenced the partial gene regions of 28S (113 bp) and *COI* (376 bp). The partial sequences were edited and connected by using CLC DNA Workbench 5 (CLCbio, Aarhus, Denmark). Sequence alignment was performed with the Clustal W program as reported by Thompson *et al.* (1994), with manual modification, and the sequences were translated into MEGA 7 (Kumar *et al.*, 2016). Substitution models (GTR + Gamma) for different partitions were selected using Kakusan 4 (Tanabe, 2011) on the basis of Akaike's information criterion (AIC) for the method of maximum likelihood (ML). Usage of combined sequence data increases the accuracy of phylogenetic

analysis (Murphy *et al.*, 2007). To improve tree accuracy, Kakusan 4 is able to calculate multiple genetic regions at the same time (Tanabe, 2007). Maximum likelihood analysis based on the analyzed 28S and *COI* sequence data from the parasitoid wasps collected was performed with 1000 bootstrap replicates. The tree was visualized and saved with the program FigTree ver. 1.4.3 (Rambaut, 2016). Further analyses were performed with Arlequin 3.5.1.3 (Excoffier & Lischer, 2010). To determine the degree of genetic differentiation among species and strains, analysis of molecular variance (AMOVA) and pairwise *Fst* values were calculated. *Fst* values and their significance were calculated by using permutation tests between pairs of *T. gerriphagus* and each strain, or between pairs of strains.

Parasitism of *Tiphodytes* to egg of water strider

Usage of an egg of water striders as host is a character of the genus *Tiphodytes* (Marchal, 1900; Nummelin *et al.*, 1988; Spence, 1986; Mineo *et al.*, 2009, 2010). Laboratory experiment was conducted to confirm whether species and strains of *Tiphodytes* developed completely from egg to adult in an egg of the water strider. Eggs of *Metrocoris histrio* (White) were used as host because they were easily reared in our laboratory. *Metrocoris histrio* were collected from Gifu in 1999–2000 and they were reared in a parasitoid-free room (14:10 h photoperiod; temperature kept at 25 °C). *Metrocoris histrio* were kept in aquaria (35 cm × 25 cm × 6 cm high, water-filled to 5 cm deep) and given frozen insects as food. The water in the aquaria was exchanged at least once a week. To acquire the eggs of *M. histrio*, wooden chopsticks were provided as oviposition substrates. The chopsticks with eggs of *M. histrio* were used as egg mass to collect parasitoid wasps at ponds or rivers in Hashima, Sekigahara and Tarui in Gifu from 1999 to 2000. YPT with detergent were also used to collect parasitoid wasps at waterside of ponds or rivers in Hashima, Sekigahara, and Tarui in Gifu from 1999 to 2000. The parasitoid wasps collected in YPT were immediately washed with water to prevent their death by detergent.

Parasitoid wasps collected were kept in small glass cylinders (diameter 2 cm × height 5 cm) containing cotton with water to maintain moisture. In the laboratory, a female parasitoid wasp was moved to another small glass cylinder with fresh eggs of *M. histrio* and allowed to attack them. After 1 day, all eggs were removed from the small glass cylinder and reared in it until parasitoid wasps have emerged. After the emergence of parasitoid wasps, we examined their morphology to identify the species or strains.

Results

Morphological observation

Some of the dry specimens of parasitoid wasp collected were confirmed by morphological observation as being of the genus *Tiphodytes*. Dry specimens of the genus *Tiphodytes* identified were categorized into *T. gerriphagus* (Fig. 1) and five groups by morphological observation (Table 5). In the present study, we called the five strains except for *T. gerriphagus* as strain 1–5 with temporary names. Strains 1–5 can be distinguished from the described *Tiphodytes* species as follows. *Tiphodytes gerriphagus* and strain 1 form a unique group having the clear wings, which are equal to body length and relatively shorter than those of the other strains. Strain 1 is quite similar to *T. gerriphagus*, but the strain 1 has the crenate occipital carina, notauli indicated by deep hole and more or less elongated T1. Strain 2 is quite similar to *T. godavari* (Mineo *et al.*, 2009). However, strain 2 is distinguishable from *T. godavari* by having slender A3 in the female, about 3.5 times as broad as length (the latter 2.5), and very short post marginal vein, which is 1/4 of stigmal vein length (the latter 1/2 of stigmal vein). Strain 3 is distinguishable from the other species and strains, by the short and broad clava in the female, it is more than 0.45 times as broad as length (less than 0.4 times in the others). Strain 4 is also very similar to *T. godavari* and strain 2, but discriminated by having vertical central keel on frons and relatively short A3–A5 in the female, about 2 times as long as width (the others A3–A5 about 3 times as long as width). Strain 5 is distinguishable from the other species and strains, by two unique character states: the female antennal clava lanceolate and the body covered with dense hairs.

DNA analysis

A phylogenetic tree based on 28S (113 bp) and COI (376 bp) sequence data analyzed from individuals of *Tiphodytes* identified was estimated by using ML supported by bootstrap (Fig. 2). The nodes of *T. gerriphagus* (100%) and strain 1–5 (100%) were well supported by bootstrap in the ML tree. The *Fst* values calculated by AMOVA of 28S and COI sequence data analyzed from individuals of *Tiphodytes* identified

were significant difference at $P < 0.05$ between pairs of *T. gerriphagus* and each strain, or between pairs of strains ($P < 0.001$; 28S: *Fst* = 1, $P < 0.001$, COI: *Fst* = 0.9714). Pairwise *Fst* values of strain 4–5 in 28S sequence data were not significant difference at $P < 0.05$ except for strain 4/strain 1–2 and strain 5/strain 1–2 and 4, and the values of COI sequence data were significant difference at $P < 0.05$ except for strain 4/strain 2 and strain 5 (Tables 3–4).

Habitats

The habitats of *T. gerriphagus* were ponds (Table 2). The habitats of strain 1 were ponds and rivers, whereas those of strain 2–5 were rivers.

Parasitism of *Tiphodytes* to egg of water strider

Table 6 shows the result of laboratory experiment that conducted to confirm whether species and strains of *Tiphodytes* completely developed from egg to adult in an egg of water strider. *Tiphodytes gerriphagus* and all strains were developed successfully in the eggs of *M. histrio*.

Discussion

Precise identification of ambiguous species, in particular for minute insects, is the first step in any ecological study. If taxonomically misidentified species names are used, it leads to serious confusion in future studies, as shown by De Mendonca & Ribeiro (2011). To solve this problem, a combination of morphological observation and DNA analysis has been developed.

First, we confirmed by morphological observation whether the parasitoid wasps collected in YPT were of the genus *Tiphodytes*. Then, in laboratory experiment, we confirmed that species and strains of the genus *Tiphodytes* identified were developed successfully in the eggs of *M. histrio*. This was important, because the usage of an egg of water striders as host is a character of the genus *Tiphodytes* (Marchal, 1900; Nummelin *et al.*, 1988; Spence, 1986; Mineo *et al.*, 2009, 2010).

Next, individuals identified as the genus *Tiphodytes* were compared with known species of the genus. Except for *T. gerriphagus*, which is the only recorded species in Japan (Ito, 1980; Hirayama & Kasuya, 2009, 2010, 2015), five strains were morphologically different from the known species.

In our DNA analysis, the *Fst* values calculated by AMOVA and the pairwise *Fst* values of 28S and COI sequence data analyzed from *Tiphodytes* identified indicated genetic differentiation between pairs of *T. gerriphagus* and each strain, and between pairs of strains, because these values were one, or close to one. Five strains are distinguishable by morphological observation, and the strains were well supported by bootstrap

Table 5. Morphological characters and habitats of *Tiphodytes gerriphagus* and other strains.

<i>Tiphodytes</i> spp.	Body length (mm)	Female clava	Occipital carina	Central keel	Fore wing/body length	Fore wing width/fore wing length	Wings color	Post-marginal vein/stigmal vein	Ciliae / width of fore wing	Notauli	Tergite 1 length/mesoscutum length	Tergite 1 width/length	Tergite 2	Habitat
<i>T. gerriphagus</i>	1.1	spindler	complete	absent	1	0.30	clear	0	1/4	shallow foveolae	0.4 - 0.5	1.4 - 1.6	glabrous	pond
Strain 1	1.1	spindler	crenate	absent	1	0.30	clear	0	1/4	deep foveolae	0.6	0.9	glabrous	pond and river
Strain 2	1.1	spindler	complete	absent	>1	0.25	irufusate	1/4	2/5	foveolae	0.5	1.5	glabrous	river
Strain 3	1.1	broad, ellipsoidal	complete	absent	>1	0.26	irufusate	2/3, pale	1/4	short grooves	0.6	0.8	glabrous	river
Strain 4	1.1	spindler	complete	present	>1	0.30	irufusate	2/3	1/4	foveolae	0.4	1.5	glabrous	river
Strain 5	1.2	lanceolate	complete	absent	>1	0.25	irufusate	0	1/3	short grooves	0.4-0.5	0.9	hairy	river

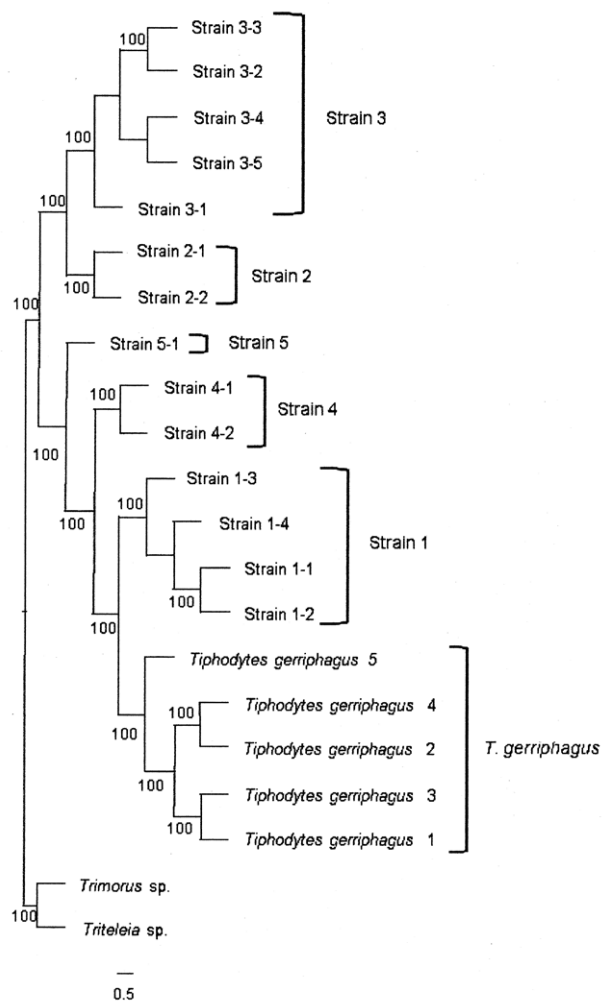


Fig. 2. Maximum-likelihood tree based on the 28S r DNA and COI gene of the genus *Tiphodytes*. Bootstrap (1000 replicates) > 50% are shown above the branches. Strains numbers indicate the group which corresponds with the article. The bar indicate substitution.

in ML tree. The pairwise *Fst* values indicated genetic differentiation between pairs of *T. gerriphagus* and each strain, or between pairs of strains.

Based on results of these morphological observation and DNA analysis we found five previously unrecognized strains, which probably include separate species. Morphological observation and DNA analysis may suggest the need for a further study on the taxonomy of Japanese *Tiphodytes*.

When a specimen is morphologically different from the previous described species, it is often difficult to determine whether the morphological characters are species-dependent differences. In this study, we categorized specimens of parasitoid wasps into *T. gerriphagus* and five strains by their morphological characters. Since the categorization was supported by DNA analysis, the morphological differences among strains would be explained by the phylogenetic relationships. Hence, the combination of morphological observation and DNA analysis is expected to be a powerful tool for the taxonomic study of *Tiphodytes*. For the taxonomic revision of Japanese species, it is needed to survey in sites of various localities.

Table. 6. Number of *Tiphodytes* emerged from *M. histrio* eggs in laboratory experiment.

<i>Tiphodytes</i> spp.	<i>Metrocoris histrio</i>
<i>T. gerriphagus</i>	2 ♀, 1 ♂ (26)
Strain 1	1 ♂ (67)
Strain 2	25 ♀, 30 ♂ (170)
Strain 3	3 ♀, 11 ♂ (116)
Strain 4	1 ♀, 9 ♂ (10)
Strain 5	3 ♀, 1 ♂ (31)

Numbers in parentheses indicate the number of *M. histrio* eggs used in laboratory experiment

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