RESEARCH ARTICLE



# Toxares koreanus sp. nov. – a new Toxares species from South Korea (Hymenoptera, Braconidae, Aphidiinae)

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Academic editor: Jose Fernandez-Triana | Received 22 March 2022 | Accepted 30 June 2022 | Published 31 August 2022

https://zoobank.org/A6251FB8-8322-4C3B-9B17-6DC5211CDEE8

**Citation:** Kim S, Tomanović Ž, Petrović A, Čkrkić J, Lee G, Lim J, Kim H (2022) *Toxares koreanus* sp. nov. – a new *Toxares* species from South Korea (Hymenoptera, Braconidae, Aphidiinae). Journal of Hymenoptera Research 92: 185–198. https://doi.org/10.3897/jhr.92.84146

#### Abstract

The genus *Toxares* Haliday, 1840 is a small taxon of Aphidiinae, consisting four valid species in the world. One *Toxares* species is recorded as new to science from South Korea, in this study. Descriptions and illustrations of the new species, *T. koreanus* **sp. nov.**, are provided, together with their mitochondrial *cytochrome c oxidase subunit I (COI)* and D2 region of the nuclear gene for 28S rRNA (28S) sequences. The phylogenetic tree reconstructed using a combination of *COI* and 28S revealed the phylogenetic position of the genus *Toxares* within Aphidiinae.

#### **Keywords**

DNA barcoding, parasitoid wasps, phylogenetics, systematics, taxonomy

### Introduction

The genus *Toxares* Haliday, 1840 is a small genus of Aphidiinae with four known species from the Holarctic. *Toxares deltiger* (Haliday, 1833) was the first species to be described from the genus. For a long time, it was only known in Europe, but it has

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been recorded from the USA (Pike et al. 2000), Turkey (Tomanović et al. 2008) and several Asian countries (Korea, Kazakhstan, Pakistan, China, India) (Choi et al. 2017; Davidian 2018, 2020). Takada (1965) described Toxares shigai Takada, 1965 from Japan, and later Shuja-Udin (1974) described two additional species, Toxares zakai Shuja-Udin, 1974 and Toxares macrosiphohagum Shuja-Udin, 1974 from India. All known Toxares species were rarely sampled, and most records came from traps and net sweeping, and consequently without evidence about its aphid hosts. Nevertheless, a part of the records came from reared aphid colonies and available literature data, and it is confirmed that aphid hosts of Toxares species belong to the tribes Macrosiphini and Aphidini including some pest aphids (e.g. Metopolophium dirhodum (Walker) (Powell 1980, 1982; Dean et al. 1981; Starý et al. 1981; Höller et al. 1993), Sitobion avenae (Fabricius) (Dean et al. 1981; Powell 1982; Cameron et al. 1984), Acyrthosiphon pisum (Harris) (Cameron et al. 1984), Myzus persicae (Sulzer) (Mackauer 1968; Starý and Ghosh 1975, 1983; Marsh 1979; Hofsvang and Hågvar 1983), Aphis craccivora (Koch) (Raychaudfuri et al. 1990), Schizaphis rotundiventris (Signoret) (Starý and Ghosh 1983) and Rhopalosiphum nymphaeae (L.) (Starý and Ghosh 1983).

Based on the forewing venation being related to braconid ancestors, the genus *Toxares* is classified within the Ephedrini tribe (Mackauer 1961), which has sometimes been considered basal within Aphidiinae (Belshaw and Quicke 1997; Sanchis et al. 2000; Derocles et al. 2012). Some other studies showed that the tribe Praini is basal (Smith et al. 1999). However, there is very little evidence about molecular data of *Toxares* species, and its phylogenetic position is still unknown. Derocles et al. (2012) determined that the phylogenetic position of *Toxares deltiger* is between Ephedrini and Praini based on sequences of the *Cytochrome c Oxidase subunit I* (*COI*) gene. Ye et al. (2017) analysed molecular markers for identification of primary parasitoids of cereal aphids. Within their analysis, *T. deltiger* clustered as a sister group of the Trioxini tribe based on *COI* sequences and as a sister group of Aphidiini based on *16S* ribosomal RNA (Ye et al. 2017).

The aim of this study is to present additional knowledge about the diversity of *Toxares* species. After initial research of Korean aphid parasitoid fauna, we recognized a new *Toxares* species which is herein described and diagnostified using morphological and molecular characters. We also analysed phylogenetic relationships among genera *Toxares*, *Ephedrus* Haliday, 1833 and *Praon* Haliday, 1833 and discussed the phylogenetic position of the genus *Toxares* within Aphidiinae.

### Material and methods

### Specimen collection and morphological analysis

Specimens were collected by Malaise trap in a deciduous forest habitat (mostly *Quercus* spp.) in Mt. Beophwa which is about 450 m.a.s.l. *Rosa multiflora*, *Cirsium japonicum*, and *Urtica thunbergiana* were the dominant plant species. Two specimens were slide-mounted with Hoyer medium and one preserved in 70% ethanol. External structure

was studied and measurements taken with a LEICA DM LS phase-contrast microscope. Morphological terminology used in this paper regarding diagnostic characters is based on that of Sharkey and Wharton (1997).

#### Molecular analysis

DNA extraction was performed using a LaboPass Tissue Kit (COSMOgenetech, Korea) following the manufacturer's protocol. In order to conserve morphologically complete voucher specimens, the DNA extraction method was slightly modified from the 'nondestructive method' by Favret (2005) and 'freezing method' by Yaakop et al. (2009). In the original protocol, the sample was crushed and then soaked in 180 µl of TL buffer + 20 µl of proteinase-K, followed by three hours of incubation at 55 °C. In the slightly modified DNA extraction methods, samples with all specimens were soaked in 180 µl of TL buffer + 20 µl of proteinase-K without destroying the sample, followed by 10 minutes incubation at 55 °C and kept in a freezer at -22 °C overnight. After that the general protocol was used for the remaining steps. The target site for molecular identification was the front partial region of mitochondrial COI, amplified using the primers LCO1490 (forward) 5'-GGTCAACAAATCATAAAGATAT-TGG-3' and HCO2198 (reverse) 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al. 1994). The molecular marker used for comparing with other Toxares species and species of Ephedrus and Praon was the D2 region of the nuclear gene for 28S rRNA (28SrDNA), amplified using primers 28SD2f (forward) 5'-AGAGAGAGTTCAAGAGTA-CGTG-3' (Belshaw and Quicke 1997) and 28SD2r (reverse) 5'-TTGGTCCGTGTTTCAAGACG-GG-3' (Campbell et al. 1993). We used heterogenous F/R primers as referred to by Tomanović et al. (2018).

Polymerase chain reaction (PCR) amplification of *COI* and 28S was conducted by using AccuPower PCR PreMix (Bioneer Corp., Daejeon, Korea) in 20 µl of a reaction mixture consisting of 3 µl of DNA extract, 2 µl of primer, and 15 µl of H<sub>2</sub>O. Thermal profile for *COI* was as follows: denaturation for 5 min at 95 °C; 38 cycles of 20 s at 95 °C, 30 s at 45 °C, and 40 s at 72 °C; and final extension at 72 °C for 5 min. Thermal profile for *28S* was as follows: denaturation for 3 min at 95 °C; 32 cycles of 30 s at 95 °C, 30 s at 48 °C, and 30 s at 72 °C; and final extension at 72 °C for 10 min. The PCR products were tested by electrophoresis on agar gel and if a band existed, we commissioned Bionocs (Korea) for sequencing and purification.

Sequences were edited with FinchTV ver. 1.4.0 (www.geospiza.com), aligned with CLUSTAL W integrated in MEGA X (Kumar et al. 2018), and trimmed to lengths of 642 bp (*COI*), and 476 bp (*28S*). Sequences are deposited in GenBank under accession numbers: ON007269–ON007271 (*COI*), ON003419–ON003421 (28S). Additional sequences from GenBank (Fig. 3) were used for phylogenetic analysis.

Average genetic distances were calculated using MEGA X and Kimura's two-parameter method of base substitution (K2P, Kimura 1980) (Table 1).

MEGA X was used to construct phylogenetic trees based on each gene used in the study, as well as a combined tree employing concatenated sequences of both genes.

Phylogenetic relationships were reconstructed using Maximum Likelihood (ML) and Maximum Parsimony (MP) methods.

	T. koreanus (JS1)	T. koreanus (JS1-1)	T. koreanus (JS1-2)	T. deltiger	E. helleni	E. nacheri	E. persicae	E. plagiator	P. abjectum	P. bicolor	P. dorsale	P. yomenae	V. canescens
T. koreanus (JS1)		0.00	0.00	0.14	0.21	0.21	0.22	0.21	0.16	0.16	0.17	0.19	0.29
		0.00	0.00	0.05	0.20	0.22	0.20	0.23	0.19	0.19	0.19	0.19	0.38
T. koreanus (JS1-1)	0.00		0.00	0.14	0.21	0.21	0.22	0.21	0.16	0.16	0.17	0.19	0.29
			0.00	0.05	0.20	0.22	0.20	0.23	0.19	0.19	0.19	0.19	0.38
T. koreanus (JS1-2)	0.00	0.00		0.14	0.21	0.21	0.22	0.21	0.16	0.16	0.17	0.19	0.29
_ · · ·				0.05	0.20	0.22	0.20	0.23	0.19	0.19	0.19	0.19	0.38
T. deltiger	0.09	0.10	0.10		0.23	0.22	0.20	0.23	0.18	0.19	0.18	0.20	0.31
					0.18	0.21	0.18	0.21	0.19	0.18	0.18	0.18	0.38
E. helleni	0.21	0.21	0.21	0.21		0.10	0.20	0.09	0.22	0.21	0.22	0.23	0.29
<b>F</b> 1 ·	0.01	0.21	0.01	0.21	0.00	0.06	0.05	0.06	0.22	0.22	0.22	0.23	0.39
E. nacheri	0.21	0.21	0.21	0.21	0.08		0.22	0.03	0.23	0.21	0.21	0.21	0.2/
E	0.21	0.21	0.21	0.20	0.12	0.16	0.08	0.00	0.21	0.22	0.22	0.21	0.41
E. persicae	0.21	0.21	0.21	0.20	0.15	0.16		0.22	0.22	0.21	0.22	0.22	0.32
E planiator	0.21	0.21	0.21	0.22	0.08	0.02	0.16	0.08	0.20	0.20	0.20	0.21	0.30
L. plugiaioi	0.21	0.21	0.21	0.22	0.08	0.02	0.10		0.24	0.21	0.22	0.21	0.42
P abjectum	0.17	0.17	0.17	0.17	0.22	0.23	0.21	0.23	0.22	0.22	0.22	0.22	0.12
1. 40 / 00 / 00	0.17	0.17	0.17	0.17	0.22	0.25	0.21	0.25		0.00	0.00	0.02	0.42
P. bicolor	0.17	0.17	0.17	0.18	0.22	0.22	0.21	0.22	0.04	0.00	0.03	0.08	0.29
	,	,	,								0.00	0.02	0.42
P. dorsale	0.17	0.17	0.17	0.18	0.22	0.22	0.21	0.22	0.04	0.02		0.08	0.28
												0.02	0.42
P. yomenae	0.18	0.18	0.18	0.19	0.22	0.22	0.22	0.22	0.06	0.05	0.05		0.28
-													0.44
V. canescens	0.32	0.32	0.32	0.33	0.32	0.32	0.33	0.32	0.361	0.34	0.34	0.34	

**Table 1.** Genetic distances (K2P) between analysed Aphidiinae species based on *COI* (bold) and *28S* (upper right) and on both genes combined (lower left).

## Results

### Description of the new species

*Toxares koreanus* Tomanović, Kim & Petrović, sp. nov. https://zoobank.org/82B185A3-71D2-4E6B-B84B-AC887FC14140

**Diagnosis.** Toxares koreanus sp. nov. morphologically resembles *T. shigai* in having elongated flagellomere 1 ( $F_1$ ), which is clearly longer than flagellomere 2 ( $F_2$ ) and elongated petiole at the spiracles level. However, *T. koreanus* sp. nov. is easily distinguished from *T. shigai* in the shape of petiole (petiole with parallel sides in *T. koreanus* sp. nov., while laterally expanded and longitudinally striated in *T. shigai*), yellow colored  $F_1$ – $F_3$  and even a yellowish base of  $F_4$  in *T. koreanus* sp. nov., while light brown colored  $F_1$ – $F_3$  in *T. shigai*. Also, *T. koreanus* sp. nov. morphologically resembles *T. macrosiphophagum*, but differs in more elongated  $F_1$  which is clearly longer than  $F_2$ , more elongated

petiole, and color of basal flagellomeres (yellow colored  $F_1-F_3$  and yellow base of  $F_4$  in *T. koreanus* sp. nov., and yellowish  $F_1$  and  $F_2$  in *T. macrosiphophagum*).

**Description. Female** (Fig. 1, Suppl. material 1: Fig. S1): *Head* (Fig. 1A) rounded, bearing sparse setae. Eyes large and oval. Tentorial index (tentoriocular line/intertentorial line) 0.30–0.36. Clypeus with about 10 long setae. Malar space equal to 0.20 of longitudinal eye diameter. Mandible bidentate, with 6–7 setae on the outer surface. Maxillary palps with four palpomeres, labial palps with three palpomeres. Antenna 17–18 segmented (17, 2 $\bigcirc$ ; 18, 1 $\bigcirc$ ), flagellate (Fig. 1B). Flagellomere 1 (F<sub>1</sub>) (Fig. 1C) clearly longer than F<sub>2</sub> (F<sub>1</sub>/F<sub>2</sub> length 1.1–1.2) and 3.75–4.00 times as long as its maximum width at the middle. F<sub>1</sub> with 2–3 and F<sub>2</sub> with 3–5 longitudinal placodes (Fig. 1C). Flagellomeres covered uniformly with semi-erect setae subequal to antennal segments diameter.

*Mesosoma.* Mesoscutum smooth, rounded, with mid pit in the middle posterior part. Notaulices distinct in very short ascendent portion of anterolateral margin, with two rows of long setae along the dorsolateral part of mesoscutum (Fig. 1D). Scutellum elongated, bearing 6–7 long setae in the central part. Scutellar sulcus divided into equal halves. Propodeum (Fig. 1E) areolated with large central areola. Upper propodeal areolas with 5–7 long setae and lower areolas with 1–4 long setae on each. Forewing (Fig. 1F) densely pubescent, with long marginal setae. Pterostigma elongated, 6.35–6.7 times as long as its width, subequal to R1 vein (Fig. 1F). Forewing 2RS vein shorter than 3RSa vein (2RS/3RSa = 0.55) and 3RSa shorter than 3RSb vein (3RSa/3RSb = 0.73).

*Metasoma*. Petiole (Fig. 1G) slightly rugose and convex dorsally, with lateral depression at level of prominent spiracular tubercles. Petiole length 2.80–2.86 times its width at the base of spiracles, with 5–6 long setae along each side (Fig. 1G). Ovipositor sheath deltoid shaped (Fig. 1H).

### Body length: about 1.70-2.20 mm.

**Coloration.** General body color light brown to yellow. Scape, pedicel and  $F_1-F_3$  yellow,  $F_4$  basally light brown, remaining antennal parts brown. Mouthparts yellow. Head brown. Mesoscutum light brown to brown. Propodeum light brown. Legs yellow with brown apices. Petiole yellow to light brown, other metasomal terga light brown. Ovipositor sheath yellow.

**Male** (Fig. 2): Antenna 19-segmented with shorter flagellomeres (Fig. 2A).  $F_1$  about 2.60 times as long as wide and longer than  $F_2$  (Fig. 2B). Number of longitudinal placodes on  $F_1$  and  $F_2$ , 3 and 5, respectively. Maxillary palps with four palpomeres, labial palps with three palpomeres. Pterostigma shorter than in female and about 4.7 times as long as wide. Mesosoma with small mid pit . Petiole shorter than in female and about 2.55 times longer than width at spiracles level. Male genitalia (Fig. 2C). Body generally darker than in female. Scapus and pedicel light brown.  $F_1$  yellow, remaining antennal parts brown. Legs yellow to light brown with dark apices. Petiole and first half of metasomal terga light brown, remaining part of metasoma brown. Legs and mouthparts light brown.

**Etymology.** The name of the new species is derived from Republic of Korea where it was found.



**Figure 1.** *Toxares koreanus* sp. nov., female **A** head **B** antennae **C** flagellomere  $F_1-F_4$  **D** mesoscutum **E** propodeum **F** petiole **G** ovipositor **H** forewing.

**Specimens examined.** *Holotype*: KOREA • 1  $\bigcirc$ ; Mt. Beophwa, San 128-1, Wolgok-ri, Cheoncheon-myeon, Jasnsu-gun, Jeollabuk-do; 35°42'07.6"N, 127°31'54.7"E; collected by Malaise trap: 06.V–24.V.2021; leg. Yeonghyeok Yu, Sangjin Kim, JuHyeong Sohn, Yunjong Han, Gyeongyeon Lee. Holotype deposited in National Institute of Biological Resources, Incheon, Republic of Korea slide mounted.

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**Figure 2.** *Toxares koreanus* sp. nov., male **A** antennae **B** flagellomere  $F_1$  and  $F_2$  **C** mesoscutum **D** propodeum **E** petiole **F** ovipositor **G** forewing.

**Paratypes:** KOREA • 1 ♂; Mt. Beophwa, San 128-1, Wolgok-ri, Cheoncheonmyeon, Jasnsu-gun, Jeollabuk-do; 35°42'07.6"N, 127°31'54.7"E; collected by Malaise trap: 06.V–24.V.2021; leg. Yeonghyeok Yu, Sangjin Kim, JuHyeong Sohn, Yunjong Han, Gyeongyeon Lee. Paratype slide mounted and deposited in National Institute of Biological Resources, Incheon, Republic of Korea. Additional material. KOREA • 2  $\bigcirc$ ; 1  $\bigcirc$ , Mt. Beophwa, San 128-1, Wolgok-ri, Cheoncheon-myeon, Jasnsu-gun, Jeollabuk-do; 35°42'07.6"N, 127°31'54.7"E; collected by Malaise trap: 06.V–24.V.2021; leg. Yeonghyeok Yu, Sangjin Kim, JuHyeong Sohn, Yunjong Han, Gyeongyeon Lee • 1  $\bigcirc$ , same locality; collected by Malaise trap: 24.V–02.VI.2021; leg. Yeonghyeok Yu, Sangjin Kim, JuHyeong Sohn, Yunjong Han, Gyeongyeon Lee. Specimens deposited dry and immersion-mounted in Kunsan National University, Jeollabuk-do, Republic of Korea.

**Molecular analysis.** Obtained phylogenetic trees reconstructed based on *COI*, 28S and the combination of both genes showed identical topology, and the tree based on the combination of both genes is shown on Fig. 3. *Toxares koreanus* sp. nov. groups with the only other *Toxares* species used in the analysis, while this clade is sister to the clade of *Praon* species. *Ephedrus* species basally form a separate clade on the tree.

Calculated genetic distances (Tables 1, 2) also indicate closer relatedness between *Toxares* and *Praon* than between *Toxares* and *Ephedrus*.

### Discussion

The genus *Toxares* is considered as one of the most basal within the subfamily Aphidiinae, classified within the tribe Ephedrini (Mackauer 1961), and sharing a braconid ancestral wing venation pattern with species of the genus *Ephedrus*. Except for the forewing venation pattern as a clear plesiomorphy, the newly described species, along with other congeners (e.g. *T. deltiger*), shares additional plesiomorphic character states, such as a large number of placodes on  $F_1$  and  $F_2$ , areolated propodeum, and 4-maxillary and 3-labial palpomeres. On the other hand, the elongated flagellomeres and petiole represent apomorphic characters (Tomanović et al. 2006). *Toxares koreanus* sp. nov. also possesses a small mid pit on the mesoscutum. This is a unique character present only in some *Ephedrus* species from the subgenus *Fovephedrus* (Chen 1986; Kocić et al. 2019), as well as in all known *Toxares* species. *Toxares koreanus* sp. nov. along with other congeners (e.g. *T. deltiger*) possesses a divided scutellar sulcus (Fig. 1D), a character state present in the subgenus *Breviephedrus* (e.g. *E. brevis*) (Kocić et al. 2019), which supports the phylogenetic position of the genus *Toxares* within the tribe Ephedrini.

	Within group mean distances							
	COI	285	combined					
Toxares	0.07	0.02	0.05					
Ephedrus	0.14	0.06	0.10					
Praon	0.07	0.01	0.04					
	Between group mean distances (COI/ 28S/ combined)							
	Toxares	Ephedrus	Praon					
Toxares								
Ephedrus	0.21/ 0.21/ 0.21							
Praon	0.19/ 0.18/ 0.17	0.22/ 0.22/ 0.22						

Table 2. Genetic distances (K2P) between genera Toxares, Ephedrus and Praon.





*Toxares koreanus* sp. nov. is the fifth known member of the genus *Toxares* and fourth species described from Asia. Based on the currently available data about the distribution of described species, we can assume that the origin of this genus should be Far Eastern Asia. Considering the habitat and plant diversity in Far Eastern Asia, we can expect to discover additional species of the genus *Toxares*.

Molecular analysis using *COI* and *28S* supports the description of the new species. *Toxares koreanus* sp. nov. is clearly separated from *T. deltiger* by both genes (Fig. 3, Table 1), in addition to morphological differences.

Molecular markers employed in this study show some incongruence with morphological characters. While Toxares is morphologically most similar to Ephedrus, molecular data suggests the genus is closer to Praon (Fig. 3, Tables 1, 2). Calculated genetic distances between all three genera are very high, based on both genes used in the analysis (Table 2). Although those between Toxares and Ephedrus are slightly higher than those between Toxares and Praon, it is still advisable to interpret these results carefully, and use an integrative approach including biological and ecological traits when making conclusions about the relatedness of groups. The discrepancy between morphological and molecular data is a fairly common occurence in Aphidiinae research and numerous studies have shown that molecular and morphological analyses often give somewhat conflicting results (Tomanović et al. 2013, 2018; Petrović et al. 2015; Jamhour et al. 2016; Čkrkić et al. 2020). One possible solution to this ongoing dilemma could be the use of more molecular markers or increasing the number of molecular operational taxonomic units, in an effort to uncover the mechanisms underlying the differences in multi-locus determined morphological traits (Zimmerman et al. 2000; Mezey et al. 2005; Čkrkić et al. 2020) and more emphasis on functions and adaptiation of morphological characters.

Although the genus *Toxares*, as a member of Ephedrini tribe, is already considered as basal within Aphidiinae (Mackauer 1961), our molecular data do not confirm it. We believe that discoveries of more species of this poorly known genus, along with appropriate molecular studies (which will include "ancient" genera *Pseudephedrus* and *Choreopraon*) should allow us to determine the exact phylogenetic position of *Toxares*.

### Acknowledgements

This work was supported by a grant from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea (NIBR202102204). This research was also supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (2018R1D1A3B07044298). The contribution of ŽT, AP and JČ is supported by the Serbian Ministry of Science and Education (451-03-68/2022-14/200178).

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### Supplementary material I

### Figure S1

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Data type: Image (tif file)

Explanation note: Figure S1. Habitus of Toxares koreanus sp. nov., female.

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Link: https://doi.org/10.3897/jhr.92.84146.suppl1