Relationship between the introduced and indigenous parasitoids *Torymus sinensis* and *T. beneficus* (Hymenoptera: Torymidae) as inferred from mt-DNA (COI) sequences

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Abstract

The parasitoid wasps *Torymus sinensis* (Ts) and three strains of *T. beneficus* (early-spring strain: TbE; late-spring strain: TbL; autumn strain: TbA) are respectively introduced and indigenous natural enemies of the chestnut gall wasp *Dryocosmus kuriphilus*, an invasive chestnut pest. In this study, the genetic relationships among Ts and three strains of Tb from Japan, China and Korea were investigated by sequencing a portion of the mitochondrial cytochrome oxidase subunit I (COI) gene. Three distinct groups were recognized in a constructed phylogenetic tree, the first group further consists of three subgroups. Ts belongs to two subgroups, whereas TbL belongs to the remaining subgroup. TbE and TbA belong to the second group, indicating that TbL is genetically more closely related to Ts a different species than to TbE and TbA strains considered the same species. This requires a reconsideration, especially for TbL, of their taxonomical treatment. The third group consisted of an indigenous *Torymus* parasitoid that we were unable to identify morphologically, indicating that an unknown *Torymus* parasitoid that is both genetically and morphologically different from the Ts and the Tb strains exploits *D. kuriphilus* in Japan.

Key words: *Torymus sinensis*; *Torymus beneficus*; cytochrome oxidase subunit I gene (COI); molecular phylogeny

INTRODUCTION

The parasitoid wasps *Torymus sinensis* Kamijo (abbreviated Ts) and *T. beneficus* Yasumatsu et Kamijo (Tb) are, respectively, introduced and indigenous natural enemies of the chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu, an invasive chestnut pest. Ts from Hebei Province in China was first released in Kumamoto and Ibaraki Prefectures in 1982 (Murakami, 1997) and has subsequently been released at various other sites in Japan (Shirai et al., 1999). It has up to now been believed that there are three Tb strains that have different emergence periods (Ôtake, 1987; Murakami, 1981, 1988): tentatively designated in this study as the “early-spring strain” (abbreviated TbE), the “late-spring strain” (TbL), and the “autumn strain” (TbA). Other than their emergence periods, little ecological information is available on the three strains of Tb. The adults of Ts and Tb have similar characteristics in external morpholo-

gies and the males are indistinguishable (Ôtake, 1987). Only the adult females of the two species can be discriminated on the basis of the ratio of the ovipositor sheath length to the thorax length (O/T ratio) (Ôtake, 1987). Ts has a larger O/T ratio than Tb (Moriya et al., 1992).

Although approximately 480 *Torymus* species have been described throughout the world (Grisell, 1995), their systematics and ecological traits, such as host species and emergence period are not well understood. Further, there are some unknown *Torymus* parasitoids morphologically unidentified. For example, some *Torymus* parasitoids collected from galls of *D. kuriphilus* in central Japan could not be categorized as Ts or Tb on the basis of the O/T ratio (Yara, unpublished data). However, few attempts have been made to identify these morphologically unknown *Torymus* parasitoids in chestnut trees (Yara et al., 2000).

In addition to the O/T ratio, an isozyme marker (malic enzyme) has been used to discriminate be-

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tween Ts and Tb (Izawa et al., 1992, 1995; Toda et al., 2000; Yara et al., 2000). None of the isozyme markers, including malic enzyme, however, can discriminate between Ts and TbL (Izawa et al., 1996; Shirai et al., 1999). Recently, many insect species have been analyzed using DNA sequence data and genetic and phylogenetic relationships among species-groups of morphologically close species have been revealed (Caterino et al., 2000).

In this study, *Torymus* parasitoids including the three Tb strains, Ts and undetermined *Torymus* parasitoids were collected from galls of *D. kuriphilus* in Japan, China and Korea. A portion of the mitochondrial cytochrome oxidase subunit I (COI) gene of each parasitoid was sequenced and a phylogenetic tree constructed in order to examine the genetic relationships between Ts and indigenous *Torymus* parasitoids, including the three Tb strains. Furthermore, using the molecular phylogenetic tree, I attempted to determine the phylogenetic position of *Torymus* parasitoids that have not been classified as either Ts or Tb on the basis of the O/T ratio.

MATERIALS AND METHODS

Insect collection. Seventy-seven adult females of *Torymus* parasitoids originating from 13 sites in Japan, Korea, and China were used for analysis (Fig. 1, Table 1). All the parasitoids, except for *Torymus* sp. 1 used as an outgroup, were collected from *D. kuriphilus* galls. Specimens were preserved in a freezer at -30 or -70°C until DNA extraction. To exclude the possibility of hybridization between indigenous *Torymus* parasitoids and introduced Ts originating in China (Moriya et al., 1992; Izawa et al., 1995), indigenous *Torymus* parasitoids were collected before introduced Ts became distributed at the collection sites in Japan (Moriya et al., 1992; Murakami, 1997; Shirai et al., 1999). For the same reason, introduced Ts was not collected in Japan, instead Ts collected from China and Korea to which Tb has not been distributed were used (Murakami, 1997).

A *Torymus* parasitoid obtained from a gall of *Celticis japonica* (Diptera, Cecidomyiidae) on a hackberry, *Celtis sinensis* var. *japonica* in Matsudo, Chiba Pref. was used as one of the outgroups ("*Torymus* sp. 1" in Table 1 and Fig. 2). This *Torymus* sp. 1 was different from any other hitherto

known *Torymus* species (Grissell, 1995) and was determined by Dr. Kamijo to be an "undescribed species" (personal communication).

Definition of Ts, Tb and unidentified *Torymus*.

The O/T ratio was calculated for each parasitoid, except for *Torymus* sp. 1, in the same manner as that described by Yara et al. (2000). According to the frequency distribution of the O/T ratios described by Moriya et al. (1992) and in Moriya's unpublished data, *Torymus* parasitoids with small O/T ratios categorized as "S" ($1.03 \leq \text{O/T} \leq 1.29$) and those with large O/T ratios categorized as "L" ($1.38 \leq \text{O/T} \leq 1.71$) were classified as Tb and Ts, respectively. For some individuals whose O/T ratios were outside the ranges of the S- or L-categories, three different categories were used: extra small O/T ratios were categorized as "XS" ($\text{O/T} < 1.03$), medium O/T ratios were categorized as "M" ($1.29 < \text{O/T} < 1.38$), and extra large O/T ratios were categorized as "XL" ($1.71 < \text{O/T}$) (Table 1). *Torymus* parasitoids in these three categories were treated as "unidentified *Torymus*" in this study.

Some individuals from Hebei Province, Liao-

Fig. 1. Collection sites of *Torymus* spp. used in this study. 1: Zunhua (Hebei Province), 2: Xingcheng (Liaoning Province), 3: Jinyang (Kyongsangnam-do), 4: Chunchon (Kangwon-do), 5: Saigo (Oki Is.), 6: Kukizaki, 7: Obuse, 8: Takamori, 9: Akitsu, 10: Ue, 11: Hirosaki, 12: Nihonmatsu, 13: Matsudo. The numbers correspond to those in Table 1.

Table 1. Collection information for *Torymus* specimens used in the study

Species	Country	Localities ^a	Emergence period	O/T ^b	N
<i>T. sinensis</i>					
	China	Zunhua, Hebei Province (1)	Mar.–Apr. 1993	L	3
	Korea	Jinyang, Kyongsangnam-do (3)	Apr. 1992	L	3
	Korea	Chunchon, Kangwon-do (4)	Apr. 2000	L	3
	Japan	Saigo, Shimane Pref. (Oki Is.) (5)	Apr. 1996	L	3
<i>T. beneficus</i> (early-spring strain)					
	Japan	Kukizaki, Ibaraki Pref. (6)	Mar.–Apr. 1988	S	17
<i>T. beneficus</i> (late-spring strain)					
	Japan	Kukizaki, Ibaraki Pref. (6)	Apr. 1988	S	9
<i>T. beneficus</i> (autumn strain)					
	Japan	Nihonmatsu, Fukushima Pref. (12)	Oct.–Nov. 1990	S	2
<i>T. beneficus</i> (emerging in spring)					
	Japan	Obuse, Nagano Pref. (7)	Apr. 1993	S	4
	Japan	Takamori, Nagano Pref. (8)	Apr. 1997	S	3
	Japan	Akitsu, Hiroshima Pref. (9)	Mar. 1991	S	2
	Japan	Ue, Kumamoto Pref. (10)	Mar.–Apr. 1993	S	3
	Japan	Hirosaki, Aomori Pref. (11)	Mar. 1993	S	1
	Japan	Nihonmatsu, Fukushima Pref. (12)	Mar. 1991	S	3
Unidentified <i>Torymus</i> ^c					
	China	Zunhua, Hebei Province (1)	Apr. 1993	M	3
	China	Zunhua, Hebei Province (1)	Mar.–Apr. 1993	S	3
	China	Xingcheng, Liaoning Province (2)	Apr. 1993	M	1
	China	Xingcheng, Liaoning Province (2)	Apr. 1993	S	1
	Korea	Chunchon, Kangwon-do (4)	Apr. 2000	S	3
	Japan	Kukizaki, Ibaraki Pref. (6)	Mar.–Apr. 1988	M	2
	Japan	Kukizaki, Ibaraki Pref. (6)	Mar. 1988	XS	4
	Japan	Hirosaki, Aomori Pref. (11)	Mar. 1993	XS	1
	Japan	Hirosaki, Aomori Pref. (11)	Apr. 1993	XL	1
	Japan	Nihonmatsu, Fukushima Pref. (12)	Nov. 1991	XS	1 ^d
<i>Torymus</i> sp. 1 ^e					
	Japan	Matsudo, Chiba Pref. (13)	May 2000	—	1

^a Numbers following the name of the localities corresponds to those in Fig. 1.

^b O/T category according to Moriya et al. (1992) and Moriya's unpublished data. XS: $O/T < 1.03$, S: $1.03 \leq O/T \leq 1.29$, M: $1.29 < O/T < 1.38$, L: $1.38 \leq O/T \leq 1.71$, XL: $1.71 < O/T$. See also text for details.

^c Specimens that could not be identified according to the O/T category and literature on distribution of *Torymus* wasps (Kamijo, 1982; Murakami, 1997).

^d Emerging in autumn.

^e A parasitoid of *Celticis japonica* (Diptera, Cecidomyiidae) obtained from galls of a leaf of *Celtis sinensis* var. *japonica*.

ning Province, and Kangwon-do were treated as unidentified *Torymus* parasitoids in spite of their S-category O/T ratios, since Tb is believed not to be distributed in China or Korea (Murakami, 1997).

The three individuals from Saigo (Oki Islands) with L-category O/T ratios used in this study were the samples that Toda et al. (2000) classified as Ts by the O/T ratio and genotype of malic enzyme. They concluded these Ts must have been present on the islands for a long time before introduced Ts were released in Tottori Pref. in 1991. Thus, the three Ts individuals were treated as “indigenous

Ts” in this study, to distinguish them from Ts being released at various sites in Japan as an introduced natural enemy.

The emergence periods of TbE and TbL do not overlap (Moriya et al., 1989), but the periods vary to some extent among years and sites. In one case, for example, TbE emerged from the latter half of March to the first half of April and TbL emerged from late April to early May (Moriya et al., 1989). Twenty-six Tb parasitoids collected from Kukizaki, Ibaraki Pref. in 1988 were divided into two types because of their two different emergence periods in

spring: 17 individuals were from March 16 to April 12 and 9 individuals were from April 27 to April 30 in 1988. The former and the latter types were treated as TbE and TbL, respectively. Other Tb parasitoids collected in spring were from different years and sites, and therefore their emergence types could not be determined. These Tb parasitoids were therefore tentatively treated as spring-emergent Tb. Tb parasitoids collected from Nihonmatsu in autumn were treated as TbA.

DNA extraction, PCR, and sequencing. DNA was extracted from a single adult using an Easy-DNATM Kit (Invitrogen) according to the manufacturer's instructions. One hundred microliters of DNA solution were finally obtained. PCR was performed in 100 μ l reaction volumes using TaKaRa Ex TaqTM (Takara Shuzo). Two primers, C1-J-1718 (5'-GGAGGATTTGGAAATTGATTAGTTCC-3') and C1-N-2191 (5'-CCCGGTAAAATTAATA-TAACTTC-3') (Simon et al., 1994) were used. The cycling conditions were as follows: samples were preheated at 94°C for 1 min, followed by 35 cycles at 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min in a Gene AmpTM PCR System 9600 (Perkin-Elmer). Target products (a fragment of COI, ca. 473 bp) were purified using a QIAquickTM PCR Purification Kit (Qiagen) and both strands directly sequenced using a Thermo SequenaseTM II dye terminator cycle sequencing kit (Amersham Pharmacia) on an ABI 373 automated sequencer (Applied Biosystems).

Based on the DNA sequences obtained, a new primer, coiT2 (5'-TCATTACCTGTTTTAGCAGG-3') was designed. To obtain an additional fragment of COI, PCR was performed using a primer pair of coiT2 and TL2-N-3014 (5'-TCCAATGCAC-TAATCTGCCATATTA-3') (Simon et al., 1994). PCR products (ca. 936 bp) were sequenced directly in the same manner.

Phylogenetic analysis of sequence data. The sequence data were aligned using Clustal W (Thompson et al., 1994). Neighbor-joining (NJ) analyses using Kimura distances (Kimura, 1980) were performed using PAUP* 4.0 beta 10 (Swofford, 2000). *Torymus* sp. 1 and *Apis mellifera* (accession number L06178) were used as outgroups. The support for each node was assessed by bootstrapping with 500 replications performed.

RESULTS AND DISCUSSION

For COI, 1,129 bp of nucleotide coding regions were determined (DDBJ/EMBL/GenBank, accession numbers AB070473 to AB070504). A total of 143 sites on the nucleotide regions were polymorphic. Thirty-one haplotypes were found among the individuals investigated. There were three distinct groups in the phylogenetic tree obtained: "Group A", "Group B" and "Group C". Group A consisted of three subgroups, "subgroup 1", "subgroup 2" and "subgroup 3" (Fig. 2).

First, the genetic relationships among Ts and the three strains of Tb (TbE, TbL, and TbA) are discussed. TbE (17 individuals collected from Kuki-zaki, highlighted in green in Fig. 2) had two haplotypes and belonged to Group B in the phylogenetic tree. TbA (two from Nihonmatsu, highlighted in yellow) had two haplotypes and also belonged to Group B. Of these individuals seven TbE (Kuki-zaki) and one TbA (Nihonmatsu) showed the same haplotype. These results indicate that TbE and TbA are not fully genetically differentiated in spite of the differences in ecological traits (emergence period). On the other hand, Ts (three from Hebei Province, three from Kyongsangnam-do, three from Kangwon-do, and three from Oki Is., highlighted in pink) had five haplotypes and belonged to subgroup 1 and subgroup 2 of Group A. TbL (nine from Kuki-zaki, highlighted in red) had four haplotypes and belonged to subgroup 3 of Group A, suggesting that TbL is more closely related to Ts considered as the different species than to TbE and TbA strains considered as the same species. This view is consistent with the fact that there are several isozyme markers that discriminate between TbE and TbL, or between Ts and TbE, whereas no isozyme marker to discriminate between Ts and TbL has yet been found (Izawa et al., 1992, 1996; Noda et al., unpublished data). It is clearly necessary to revise the taxonomical treatment of Ts and the three strains of Tb, especially TbL (Kamijo, 1982; Murakami, 1997).

The results of this study make it possible to determine the phylogenetic position of the "unidentified *Torymus*" collected from China and Korea, which could not be identified by O/T ratio. As described above, Ts formed two genetically different subgroups, i.e., subgroup 1 and subgroup 2 of Group A (Fig. 2). In subgroup 1, six individuals of

Fig. 2. Phylogenetic tree inferred from 1,129 bp sequences of the mitochondrial COI gene of *Torymus* spp. using the neighbor-joining method. *Apis mellifera* and *Torymus* sp. 1 were used as outgroups. The abbreviations for each species and strain are as follows; Ts: *Torymus sinensis* (highlighted in pink), TbE: early-spring strain of *T. beneficus* (highlighted in green), TbL: late-spring strain of *T. beneficus* (highlighted in red), TbA: autumn strain of *T. beneficus* (highlighted in yellow), Tb: *T. beneficus* emerging in spring (strain unidentified), T*: unidentified *Torymus*. One unidentified *Torymus* from Hirosaki with the XL-category O/T ratio is highlighted in blue. O/T categories are indicated in square brackets. The number of specimens are given in parentheses. Bootstrap probabilities (500 replicates) are provided on each branch when they are greater than 70%. The scale of distances is shown under the tree.

unidentified *Torymus* collected from China (five from Hebei Province and one from Liaoning Province in the S- or M-category O/T ratios) were found. Of these individuals three “unidentified” specimens from Hebei Province had the same haplotype, as did two Ts from Hebei Province. Thus, we can say from the genetic viewpoint that these unidentified *Torymus* were Ts, in spite of their smaller O/T ratios. Similarly, five other unidentified *Torymus* collected from China and Korea (one from Hebei Province, one from Liaoning Province and three from Kangwon-do with the S- or M-category O/T ratios) can be regarded as Ts because they belong to subgroup 2. Furthermore, these two subgroups appear to be different from each other in

collection site: subgroup 1 consisted of Ts collected from China only, whereas subgroup 2 consisted of parasitoids from China, Korea and Japan (Oki Is.). This prompts an interesting question as to whether the latter is distributed more widely than the former. However, further investigation is needed to answer this question.

We were also able to determine the phylogenetic position of the Tb parasitoids and most of the unidentified *Torymus* that could not be identified by O/T ratio and emergence period collected from Japan. Although the spring-emerging Tb specimens (four from Obuse, three from Takamori, two from Akitsu, three from Ue, one from Hirosaki and three from Nihonmatsu) had not had their emer-

gence types (TbE or TbL) determined, all of them belonged to Group B (Fig. 2). These individuals could be regarded as TbE because Group B included TbE and TbA. The unidentified *Torymus* emerging in spring (six from Kukizaki in the XS- or M-category and one from Hirosaki in the XS-category O/T ratios) and those emerging in autumn (one from Nihonmatsu in the XS-category O/T ratio) also belonged to Group B. Thus, it is conceivable that the former and the latter were TbE and TbA, respectively. However, we cannot genetically identify one unidentified *Torymus* with the XL-category O/T ratio collected from Hirosaki (highlighted in blue in Fig. 2). This individual solely formed Group C, indicating an indigenous *Torymus* parasitoid, which was genetically as well as morphologically different from the Ts and Tb strains, exploited galls of *D. kuriphilus* in Japan. Further investigation will be needed to genetically identify the unidentified *Torymus* parasitoids.

The O/T ratio has up to now been used as a good indicator for simply discriminating between Ts and Tb, though it has not identified several *Torymus* parasitoids exploiting *D. kuriphilus* galls in chestnut trees. The present results contradict the general belief that Ts has a larger O/T ratio while Tb has a smaller one (Kamijo, 1982; Murakami, 1997), categorized respectively as L- and S-O/T ratio in this study. As described above, the unidentified *Torymus* from China and Korea can be treated as Ts in the phylogenetic tree, in spite of their different O/T ratios categorized as M or S. In addition, the unidentified *Torymus* from Japan can be regarded as TbE or TbA, in spite of their O/T ratios categorized as M or XS. These results indicate that the O/T ratio is not a reliable marker for discrimination between Ts and Tb. Much more accurate information is likely to be obtained by using COI and other reliable molecular markers to investigate the ecological interactions of *Torymus* as well as their genetic relationships (Yara et al., 2000).

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