Discrimination of *Torymus sinensis* Kamijo (Hymenoptera: Torymidae) and *T. beneficus* Yasumatsu et Kamijo and their hybrids by allele-specific PCR

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Abstract

*Torymus sinensis* and *Torymus beneficus* (Hymenoptera: Torymidae) are, respectively, introduced and indigenous parasitoid wasps that attack the invasive chestnut gall wasp, *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae) in Japan. *Torymus beneficus* has two emergence types in spring, here designated early-spring and late-spring. It is very difficult to distinguish these *Torymus* species and emergence types of *T. beneficus* by their morphological and ecological characteristics. We designed four allele-specific primers for the internal transcribed spacer 1 region of nuclear ribosomal DNA, and developed three kinds of allele-specific PCR. These methods enabled us to distinguish the species, emergence type, and F1s. This is the first report of the use of molecular markers to distinguish *T. sinensis*, the late-spring strain of *T. beneficus*, and their F1s.

Key words: *Torymus sinensis; Torymus beneficus; internal transcribed spacer 1; ribosomal DNA; allele-specific PCR*

INTRODUCTION

*Torymus sinensis* Kamijo is a parasitoid wasp introduced into Japan to control the invasive chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu (discussed as a pest in Murakami, 1997; Moriya et al., 2003; Aebi et al., 2006; Abe et al., 2007; Cooper and Rieske, 2007; and their references). After *T. sinensis* was introduced from China into Japan and released in 1982, the damage caused by *D. kuriphilus* fell precipitously (Moriya et al., 2003).

*Torymus beneficus* Yasumatsu et Kamijo is an indigenous parasitoid which also parasitizes *D. kuriphilus*. It is morphologically very similar to *T. sinensis*; only the adult females can be discriminated empirically, by a slight difference in the length of their ovipositor sheath relative to the thorax (Ôtake et al., 1984; Ôtake, 1987; Moriya et al., 1992); the males cannot be discriminated empirically (Ôtake et al., 1984). A further complication is that *T. beneficus* has been divided into two emergence types, provisionally designated here as the early-spring and late-spring strains, according to a slight difference in their emergence periods (Ôtake, 1987; Murakami, 1988).

While the introduction of *T. sinensis* is regarded as one of the most well-known and successful cases of classical biological control in Japan, the interaction between *T. sinensis* and *T. beneficus* has received attention with regard to non-target effects of biological control (Barratt et al., 2006; Hopper et al., 2006). Field investigations based on morphological discrimination suggested displacement of *T. beneficus* by *T. sinensis* and hybridization between them (Moriya et al., 1992); however, these concerns have been only partially clarified, mainly because the discrimination of *Torymus* species and emergence types is so difficult.

Some molecular markers have been proposed as a means of partially discriminating these parasitoids [malic enzyme (EC 1.1.1.40): Izawa et al., 1992, 1996; the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA (nrDNA): Yara, 2006; cytochrome oxidase subunit I (COI) of mitochondrial DNA (mtDNA): Yara et al., 2007]. Using these markers, several authors have studied
the interaction (displacement, hybridization) between *T. sinensis* and the early-spring strain of *T. beneficus* (Izawa et al., 1996; Toda et al., 2000; Yara et al., 2000, 2007); however, no molecular markers are available to discriminate between *T. sinensis*, the late-spring strain of *T. beneficus*, and their F1 hybrids. Thus, little is known about the interactions, especially hybridization, between *T. sinensis* and the late-spring strain of *T. beneficus*, although the possibility has been suggested (Moriya et al., 1992; Yara et al., 2000).

Our objective was to develop DNA markers that could easily and clearly identify *Torymus* species, including individuals that cannot be determined by the existing molecular markers. We therefore investigated whether the ITS1 region of nrDNA is a suitable marker for discrimination. We propose a method based on an allele-specific PCR technique and evaluate its usefulness.

### MATERIALS AND METHODS

**Insects.** The wasps used in this study are listed in Table 1. *T. beneficus*, both early- and late-spring strains, and *T. sinensis* were the same as used in Yara (2006). *T. beneficus* were collected where *T. sinensis* had never ranged, in order to exclude the possibility that the specimens were hybrids (Moriya et al., 1992; Izawa et al., 1996). Consequently, one population of each *T. beneficus* strain was used in this study.

We tested F1s of a cross between the late-spring strain of *T. beneficus* and *T. sinensis*, which had been preserved in a freezer at $\leq-20^\circ$C at the National Institute of Fruit Tree Science (Moriya et al., 1992). No confirmed F1s of the cross between the early-spring strain of *T. beneficus* and *T. sinensis* were available, so we tested specimens with the FS genotype for malic enzyme, which were thought to be such hybrids (Toda et al., 2000; unpublished data) and had been stored at $-70^\circ$C at the National Institute of Fruit Tree Science.

**DNA extraction, PCR, and sequencing.** DNA was extracted from individuals with a Genomic-Prep Cells and Tissue DNA Isolation Kit (GE Healthcare) according to the manufacturer’s instructions. The extracted DNA was hydrated with 100 μl DNA hydration solution from the kit.

To amplify the ribosomal ITS1 region (including portions of the flanking 18S and 5.8S genes), two primers, CAS18sF1 (5’-TACACACCGCGCTACTA-3’) and CAS5p8sB1d (5’-ATGTGCTTCTCRAAAATGCATGTTCA-3’) (Ji et al., 2003) were used. PCR was carried out in 25 μl reaction mixtures containing 0.2 mM each dNTP, 0.2 mM each primer, 0.5 μl template DNA, and 0.65 U Taq polymerase (*TaKaRa Ex Taq*, Takara Bio Inc.), and 1×Ex *Taq* buffer (2.0 mM Mg$^{2+}$ concentration). The cycling conditions were as follows: 94°C for 1 min and 35 cycles at 94°C for 15 s, 55°C for 15 s, and 72°C for 1 min 30 s in a GeneAmp PCR System 9600 (Perkin-Elmer).

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen), and both strands were directly sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

**Allele-specific PCR.** From ITS1 sequence data (Fig. 1), we designed four allele-specific primers:

### Table 1. Collection details of specimens used in this study

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Country</th>
<th>Locality</th>
<th>Year</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Torymus beneficus</em> (early-spring strain)</td>
<td>Japan</td>
<td>Kukizaki, Inashiki, Ibaraki Pref.</td>
<td>1988</td>
<td>16</td>
</tr>
<tr>
<td><em>T. beneficus</em> (late-spring strain)</td>
<td>Japan</td>
<td>Kukizaki, Inashiki, Ibaraki Pref.</td>
<td>1988</td>
<td>13</td>
</tr>
<tr>
<td><em>T. sinensis</em></td>
<td>China</td>
<td>Zunhua, Hebei Prov.</td>
<td>1993</td>
<td>8</td>
</tr>
<tr>
<td>China</td>
<td>Xingcheng, Liaoning Prov.</td>
<td>1993</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Hybrid F1 a</td>
<td>1985</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specimens with FS genotype for malic enzyme b</td>
<td>Japan</td>
<td>Saigo, Oki, Shimane Pref.</td>
<td>1996</td>
<td>2</td>
</tr>
<tr>
<td>Japan</td>
<td>Akitsu, Toyota, Hiroshima Pref.</td>
<td>1996</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

a Obtained from crossing experiments: *T. beneficus* (late-spring strain)×*T. sinensis* (Moriya et al., 1992).
b Thought to be F1 hybrid between *T. beneficus* (early-spring strain) and *T. sinensis* (Toda et al., 2000; unpublished data).
its1_R23A (5'-CAAGACAGGGGTTGACAAAA-GAA-3'), its1_3F20T (5'-CCGTTAAGTCAAAA-CACCGT-3'), its1_R23G (5'-CAAGACAGGGGTGACAAAA-GAA-3'), and its1_3F21C (5'-CCGTTAAGTCAAAAACACCGC-3'). Two (its1_R23A and its1_3F20T) or one (either its1_R23G or its1_3F21C) of the allele-specific primers, and two nonspecific primers (CAS18sF1 and CAS5p8sB1d) were used for each allele-specific PCR. PCR was carried out in 25 μl reaction mixtures containing 1×PCR buffer (10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2), 0.2 mM each dNTP, 0.2 μM each primer, 0.5 μl template DNA, and 0.65 U Taq polymerase (TaKaRa Taq™ Hot Start Version, Takara Bio Inc.). The amplification conditions were equivalent to both touchdown PCR (Don et al., 1991) and shuttle PCR (two-temperature PCR): initial denaturation at 94°C for 1 min, 35 cycles of 15 s of denaturation at 94°C and 1 min of annealing-extension at 75 to 66°C decreasing by 1°C every cycle for the first 10 cycles, then 65°C for the following 25 cycles, and a final extension at 72°C for 10 min in a GeneAmp™ PCR System 9600 (Perkin Elmer). The PCR products were electrophoresed in 1.0% agarose gel (Agarose S; Nippon Gene) using 1×TAE buffer at 100 V for 30 min and visualized with ethidium bromide.

RESULTS AND DISCUSSION

ITS1 sequences

ITS1 and flanking sequences were determined from 31 of 50 individuals (excluding F1s and FS genotype) by direct sequencing. The most frequent sequences in T. beneficus (early- and late-spring strains) and T. sinensis were considered as typical types of each T. beneficus, and were submitted (Acc. Nos. AB446547–AB446549). The entire lengths of the ITS1 of T. beneficus (early-spring strain), T. beneficus (late-spring strain), and T. sinensis were estimated as 931, 946, and 944 bp respectively (Table 2), by comparison with the rRNA gene sequences of two Hymenoptera, Leptothorax acervorum Fabricius (Formicidae) (Acc. No. X89492) and Mesochorus sp. (Ichneumonidae) (Acc. No. AY588968). Thus, it is difficult to identify these Torymus species by agarose gel electrophoresis (1–2% concentration) of PCR products in the ITS1 region.

Inter-specific and inter-strain single nucleotide substitutions were detected at six sites. Five were variations between the early-spring strain of T. beneficus and the others (late-spring strain of T. beneficus and T. sinensis), and the other was the only variation between the late-spring strain of T. beneficus and the others (early-spring strain of T. beneficus and T. sinensis). Intra-specific and intra-strain variations were also detected (Table 2), but most were 2- or 3-base indels (insertions/deletions), and a few were nucleotide substitutions at different sites from the inter-specific and inter-strain variations; therefore, ITS1 will allow clear discrimination of these Torymus species and strains, as long as single nucleotide variations between the species and strains can be detected.

ITS1 sequences of the remaining 19 individuals could not be determined completely by direct sequencing because there were double peaks in the middle of the electropherograms, suggesting more than one ITS1 sequence type; however, the partial sequences that could be determined by direct sequencing were the same as the typical sequences,
and inspection of the electropherograms by eye suggested that most individuals had two closely similar sequences that differed slightly in the number of simple sequence repeats (e.g., (AT)₅ and (AT)₆). Furthermore, allele-specific PCR by primers based on the obtained sequence data was successful, as mentioned below, in these 19 individuals; thus, we did not clone the PCR products of the ITS1 region for sequencing.

**Allele-specific PCR**

The four new specific primers and two nonspecific primers produced the expected PCR products in each of three kinds of allele-specific PCR. Consistent results were obtained for all 56 individuals examined.

The first allele-specific PCR, using two specific (its1_R23A, its1_3F20T) and the two nonspecific (CAS18sF1, CAS5p8sB1d) primers (Fig. 2a) produced a specific ~1,000 bp amplicon and a nonspecific ~1,200 bp amplicon from late-spring *T. beneficus* and *T. sinensis* F₁s; a nonspecific ~1,200 bp amplicon from early-spring *T. beneficus* and *T. sinensis*; a specific ~300 bp amplicon from early-spring *T. beneficus* and *FS* genotypes, and *T. sinensis*, late-spring *T. beneficus*, and F₁s, although a nonspecific ~1,200 bp amplicon was detected faintly.

Two *T. beneficus* strains were collected from Kukizaki (Table 1) and their ITS1 sequence data support the design of the specific primers. On the other hand, F₁s and *FS* genotypes were not collected from Kukizaki: F₁s were obtained from crossing experiments between late-spring *T. beneficus* from Yatabe, Tsukuba, Ibaraki Pref., and *T. sinensis* from China (S. Moriya, personal communication), and *FS* genotypes were collected from Saigo and Akitsu (Table 1). Nevertheless, the specific primers produced the expected amplicons from F₁s and *FS* genotypes; thus, it is reasonable to consider that allele-specific PCR is possible even though the specific primers were based on ITS1 sequences from only a few *Torymus* spp. populations.

The results in this study allowed us to construct a key to identify the *Torymus* parasitoids and their F₁s based on allele-specific PCR of ITS1 (Table 3). It is noteworthy that we can discriminate between late-spring *T. beneficus*, *T. sinensis*, and their F₁s, because no molecular markers discriminating them

<table>
<thead>
<tr>
<th>Species</th>
<th>Female</th>
<th>Male</th>
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<tbody>
<tr>
<td><em>T. beneficus</em> (early-spring strain)</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td><em>T. beneficus</em> (late-spring strain)</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td><em>T. sinensis</em> [Hebei]</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>T. sinensis</em> [Liaoning]</td>
<td>6</td>
<td>2</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Typical TbE (931 bp)</th>
<th>TbE_S, I (936 bp)</th>
<th>Typical TbL (946 bp)</th>
<th>Typical Ts (944 bp)</th>
<th>Ts_S (944 bp)</th>
<th>Ts_F (946 or 950 bp)</th>
<th>Ts_S, I (949 bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
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<tr>
<td><em>T. beneficus</em></td>
<td>8</td>
<td>7</td>
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<td></td>
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<tr>
<td><em>T. beneficus</em></td>
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<tr>
<td><em>T. sinensis</em></td>
<td>2</td>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>Male</td>
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<td></td>
</tr>
<tr>
<td><em>T. sinensis</em> [Hebei]</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>T. sinensis</em> [Liaoning]</td>
<td>2</td>
<td>1</td>
<td></td>
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</tbody>
</table>

* TBE: *T. beneficus* (early-spring strain); TBL: *T. beneficus* (late-spring strain); Ts: *T. sinensis*. S: Nucleotide substitutions were found in typical type. I: Insertions were found in typical type.

* Including three indel patterns.
have hitherto been obtained. Furthermore, the allele-specific PCRs of ITS1 used are cost-effective, simple, and rapid, and require very basic molecular biology equipment compared with methods used for other molecular markers to study T. sinensis and T. beneficus (Izawa et al., 1992; Yara, 2006). Thus, ITS1 will be very helpful in studying interactions between all three Torymus parasitoids. We are currently investigating hybridization between T. sinensis and late-spring T. beneficus in the field using this technique. Genetic interactions between them should be clarified in the near future.

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