

*Full Length Research Paper*

# Molecular and biological characterization of *Trichogramma turkestanica* (Hymenoptera: Trichogrammatidae) which inhabits Taif governorate at the west of Saudi Arabia

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Parasitoids of *Trichogramma* (Hymenoptera: Trichogrammatidae) parasitize the eggs of many species of Lepidoptera and have been used for the biological control of numerous pest species. We collected this parasitoid from Taif governorate, KSA in summer of 2009. It is difficult to differentiate between *Trichogramma* species because of their small size and lack of differences in morphological characters. Therefore, different molecular markers were employed to characterize this species, including direct amplification of the internal transcribed spacer 2 (ITS2) of ribosomal DNA and by restriction fragment length polymorphism followed by sequencing. The results show that ITS2 region is 491 bp and indicated that this is a new strain of *Trichogramma*. We named this strain TaifKSA. From the tested restriction enzymes, only *EcoRI* and *PstI* cut the PCR product of ITS2 region. We compared the biological characteristics of the strain under investigation with other commercial strain (SQG) of the same species and no significant differences between them have been shown.

**Key words:** *Trichogramma turkestanica*, TaifKSA, molecular identification, internal transcribed spacer 2 (ITS2), restriction enzymes, biological characteristics.

## INTRODUCTION

Wasps of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) parasitize the eggs of many species of Lepidoptera and have been used for the biological control of numerous pest species in a wide range of agricultural and forestry crops throughout the world (Li, 1994; Smith, 1996). Using these parasitoids on these pests prevents the damage, and the injurious stage is larvae. These lepidopterous pests induce high damage on the economical parts of the plant which are fruits and leaves (Hassan, 1993; Hunter, 1994).

The genus *Trichogramma* comprises of more than 100 species. As an evidence, considerable variations between species exist, especially with respect to host preference,

searching capacity and tolerance to weather conditions (Hassan, 1994). The genus *Trichogramma* attacks more than 400 species in 203 genera, 44 families and 7 orders (Bao and Chen, 1989). More than 70 species of *Trichogramma* have been used all over the world (Li, 1984).

In biological control programs using parasitoids of the genus *Trichogramma*, a very important step is the identification and the use of correct species to be released in the field. The identification of these wasps is difficult due to their size (0.25 mm in length) and their identification at species level depends mainly on male genitalia. The problem is that only females can be found in many species under natural conditions. *Trichogramma* is minute and indistinguishable morphologically, further, the environmental factors influence significantly its morphology and physiology. So, identification of the wasp is problematic and its systematic needs to be clarified

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(Pinto, 1998). Investigations using the *Trichogramma turkestanica* Meyer have been previously conducted under the name *Trichogramma evanescens*, Lager in many countries (Hansen, 2000). Molecular identification of this wasp can be realized through PCR of 'species-conserved region' and rDNA-ITS2. Due to the small size and few characters available, specific identification is still difficult, particularly when sibling species need to be distinguished. In addition, the specimen preparation for morphological identification is time consuming. Recently, the identification of this minute wasp is based on DNA sequences of the internal transcribed spacer (ITS-2). This technique can allow a quick and precise *Trichogramma* species identification (Americo et al., 2001; Li et al., 2005; Li, 2007). At the species and intraspecific levels, the internal transcribed spacer 1 (ITS1) and ITS2 regions have been often used as a taxonomic tool for insect identification (Campbell et al., 1993; Hoy, 1994). The sequence and restriction analyses of the ITS2 rDNA have been described as a tool for *Trichogramma* identification as well (Kan et al., 1996; Pinto et al., 1997; Stouthamer et al., 1999). A single microsatellite marker was used to distinguish between strains of *Trichogramma cacoeciae* (Pizzol et al., 2005). The genus *Trichogramma* acquires a high specific and geographic diversity which makes it possible to select the optimal species and strains to control future pests. Studies have been done to quantify genetic variations among individuals within beneficial organism. This should lead to improved results in the biological control of pests. Concerning *Trichogramma*, genetic variability in quantitative biological traits has been investigated. Significant variations can be observed in basic biological features of *Trichogramma* reproductive strategy. Such variability provides the fundamental basis from which artificial selection programmes can be started in order to improve the efficiency of released *Trichogramma* in pest control (Wajnberg and Hassan, 1994).

According to previous knowledge, this study aim to identify *Trichogramma* inhabiting Taif governorate based on DNA sequences of the internal transcribed spacer (ITS-2). The second aim was to study the strain efficacy (emergence, developmental time, longevity, parasitism capacity/female, sex ratio) for future utilization in the field.

## MATERIALS AND METHODS

### Collection of *Trichogramma*

There are two methods to collect *Trichogramma*, the first depends on naturally laid host eggs and the second depends on artificially placed eggs (Pinto et al., 2002). We collected this parasitoid by the second method where trap-host eggs of *Ephesia kuehniella* Zeller (Lepidoptera: Pyralidae) were glued to small strips of index card and placed in trees. Different areas where vegetables (tomatoes, eggplants, cabbage, etc.) and fruit trees (citrus, grapevine, pomegranate, etc) are grown were visited to collect *Trichogramma* sp. The cards were collected after 2 to 3 days. Eggs were kept in the laboratory until adult parasitoid emerged. After that both sexes

were separated in glass tubes for biological studies.

## Molecular identification

### DNA extraction by Chelex-100

Five female individuals were ground with 100  $\mu$ l of 5% Chelex-100 and 4  $\mu$ l of proteinase K (20 mg/ml) and incubated 6 h at 56°C followed by 10 min at 95°C (Almeida and Stouthamer, 2003). The mixture was spun at 12000 g for 10 min and the supernatant was transferred to new tubes. Five microliters of the supernatant were used as PCR template.

### PCR of ITS2 region

The PCR was done in a total volume of 50  $\mu$ l. For each reaction, 5  $\mu$ l of DNA template were used, plus 45 ml of the PCR mix (5  $\mu$ l of PCR buffer, 1  $\mu$ l of dNTP's each in a 10 mM concentration), 1  $\mu$ l of the ITS2 forward and reverse primers (Campbell et al., 1993) and 0.5  $\mu$ l of TAQ polymerase (Promega). 200 pmole forward primer (5'-TGTGAA CTGCAGGACACATG-3') located in the 5.8s region and 200 pmole of the reverse primer (5'-GTCTTGCTGCTCTGAG-3') located in the 28s region were applied.

### Restriction analysis

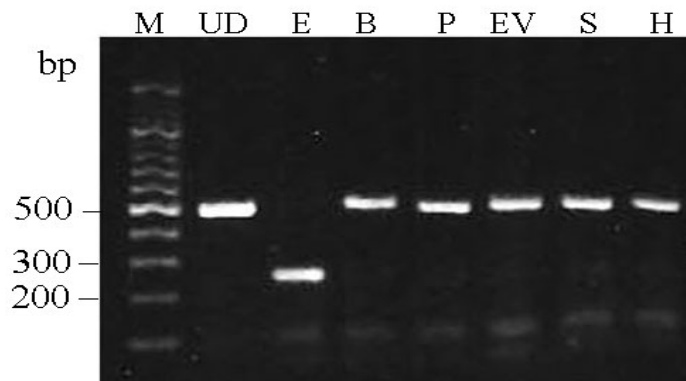
The PCR products were digested with 6 of restriction enzymes: *EcoRI*, *BamHI*, *PstI*, *EcoRV*, *SmaI* and *HindIII* in a total volume of 10  $\mu$ l, and incubated at 3°C for one hour (Americo et al., 2001). PCR products were treated with a restriction enzyme and electrophoresed on 1% agarose gel in 1  $\times$  TBE buffer and visualized by staining with ethidium bromide (Muraji et al., 2004).

### Bioinformatic and sequence alignment

Obtained PCR product was sequenced (Macrogen, <http://www.macrogen.com>) and deposited in the EST database (Accession no. HM185391). The obtained sequence for the endemic *Trichogramma turkestanica* TaifKSA strain was aligned with the top five hits of the same species in the nucleotide sequence database (Tt2, Accession no. AF043614.1; V.F, Accession no. DQ137264.1; Benavente, Accession no. DQ137263.1; CHT-3, Accession no. DQ088061.1; SQG, Accession no. DQ088062.1) using Clustalw2 (<http://www.ebi.ac.uk/Tools/clustalw2/>).

### Biological studies

In order to determine the efficiency of the endemic egg parasitoid *Trichogramma*, we compared it with the commercial strain of *T. turkestanica* SQG that has been obtained from Natural Enemies Mass Production Center, Faculty of Agriculture, Cairo University, Egypt. Artificial egg mass of *E. kuehniella* was prepared by gluing 100 to 200 eggs to a small piece of cardboard and introduced to one female and one male wasp in a Petri dish (5 cm in diameter) that contained a streak of dilute honey as a food source (McGregor et al., 1998). The cardboards were daily changed until the death of parasitoid females. This treatment was repeated 20 times for both endemic and commercial strains. We studied the developmental time, longevity, parasitism capacity/female, sex ratio and emergence rate. The experiments were done under controlled conditions of 26  $\pm$  1°C, 70 to 80% RH, and a 14L : 10D photoperiod. The males and females used in the experiments were



**Figure 1.** PCR product digestion of ITS2 region of *T. turkestanica* TaifKSA strain. M: 100 bp ladder; UD: undigested PCR product; E, B, P, EV, S, H: PCR product digested with restriction enzymes *EcoRI*, *BamHI*, *PstI*, *EcoRV*, *SmaI* and *HindIII*, respectively.

less than 12 h old. Data from all experiments were statistically analyzed using classification one-way MSTAT version 4 (1987). Significant differences among these means were carried out by LSD test ( $p \leq 0.05$ ).

## RESULTS AND DISCUSSION

### PCR product and its digestion

PCR of the ITS2 region gives identical bands of 491 bp in the commercial and the strain that inhabits Taif governorate. PCR product was digested with many restriction enzymes; six enzymes among them are shown in Figure 1. *EcoRI* cuts the fragment at 239 from the 5' end, yet it showed one band on agarose gel at about 250 bp (Figure 1). Sequencing data showed that *PstI* cuts the PCR product after 8 nucleotide from the 5' end which is difficult to be distinguished on agarose gel. Other enzymes used did not cut the fragment.

### Bioinformatic and sequence alignment

The alignment showed that TaifKSA is closer to strains CHT-3 and SQG than the other three strains. Our strain does not have the first 20 nucleotides of strains CHT-3 and SQG. There are two additions of two nucleotides (GC) and one nucleotide (C) at 162, 163 and 172 consequently in TaifKSA and in CHT-3 and SQG. The other two strains (Tt2, V.F, Benavente) did not show these additions. The new strain missed two nucleotides (GC) at 423 and 424 and another two (GG) at 428 and 429 as well as an A at 440. Also, there were two missing nucleotides at the end of the sequence at positions 490 and 491 as compared to CHT-3 and SQG (Figure 2). Chassain et al. (1988) recorded variations among the strains of *Trichogramma*. The authors also revealed that the range of variations within *Trichogramma cacaeciae*

falls within that of *Trichogramma brassicae*. Variations of both traits were correlated and strains could be easily classified according to their overall tendency to concentrate on their attacks. The adaptive importance of the foraging behavior of parasitoid insects suggests that differences reported here could reveal adaptive behavioral differentiations of natural populations. These differentiations could be in response to local variations in selective constraints, mainly those emanating from host diversity, abundance and distribution. Multiple alignment of TaifKSA sequence with *T. turkestanica* strains showed that TaifKSA has diverged before CHT-3 and SQG, however the three strains are much closer to each other and diverged later than Benavente, Tt2 and VF strains (Figure 3). It seems that TaifKSA, CHT-3 and SQG elapsed more for their divergence than Benavente, Tt2 and VF.

### Biological characteristics

Data presented in Table 1 indicate that there was no significant difference between the two examined strains of *T. turkestanica* in all tested biological activities. TaifKSA strain acquired developmental period of  $10.2 \pm 0.6$  days as compared to  $9.7 \pm 0.83$  days in SQG strain. Female longevity in TaifKSA strain was  $4.1 \pm 0.92$  as compared to  $3.7 \pm 1.23$  days in SQG. The parasitism capacity/female was  $41.8 \pm 6.61$  in TaifKSA as compared to  $38.55 \pm 8.2$  eggs in SQG. The sex ratio was  $64.7 \pm 4.56$  in TaifKSA as compared to  $62.4 \pm 5.91$  in SQG. Meanwhile, the emergence rate was slightly less in TaifKSA strain ( $92.6 \pm 7.8\%$ ) than in SQG strain ( $95.3 \pm 9.24\%$ ). On the other hand, at 25°C and 16L : 8D, Boivin and Lagacé (1999) revealed that female *T. turkestanica* lives on eggs of *E. kuehniella* for 3.5 days with a sex ratio of 80% during the first day which is higher than ours. This discrepancy could be attributed to the fact that female *T. turkestanica* produces female progenies first and this

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CHT-3      CAGCTAATTGCGCGTCAACTTGTGAA--CTGCAGGACACATGAACATCGACATTTTCAACG 59
SQG        CAGCTAATTGCGCGTCAACTTGTGAA--CTGCAGGACACATGAACATCGACATTTTCAACG 59
TaifKSA    -----TGTGAAACTGCAGGACACATGAACATCGACATTTTCAACG 40

Tt2        -----GTTTATAAAA 10
V.F.       -----GTTTATAAAA 10
Benavente  -----GTTTATAAAA 10
CHT-3      CACATTGCGGTCCACGGATCTCGTTCGCCGACCACGCCTGGCTGAGGGTCGTTTATAAAA 119
SQG        CACATTGCGGTCCACGGATCTCGTTCGCCGACCACGCCTGGCTGAGGGTCGTTTATAAAA 119
TaifKSA    CACATTGCGGTCCACGGATCTCGTTCGCCGACCACGCCGGCTGAGGGTCGTTTATAAAA 100
                *****

Tt2        ACGAACCCGACTGCTCTCTCGCAAGAGAGAGCGTTGATCTGGGCGCTCGTGTCTCTATCT 70
V.F.       ACGAACCCGACTGCTCTCTCGCAAGAGAGAGCGTTGATCTGGGCGCTCGTGTCTCTATCT 70
Benavente  ACGAACCCGACTGCTCTCTCGCAAGAGAGAGCGTTGATCTGGGCGCTCGTGTCTCTATCT 70
CHT-3      ACGAACCCGACTGCTCTCTCGCAAGAGAGAGCGTTGATCTGGGCGCTCGTGTCTCTATCT 179
SQG        ACGAACCCGACTGCTCTCTCGCAAGAGAGAGCGTTGATCTGGGCGCTCGTGTCTCTATCT 179
TaifKSA    ACGAACCCGACTGCTCTCTCGCAAGAGAGAGCGTTGATCTGGGCGCTCGTGTCTCTATCT 160
                *****

Tt2        C--TCTCTACTCTTCTTTCGAAGTGTATAGAG-AGCAGTGTGTGATACGTCGCCTCAAACG 127
V.F.       C--TCTCTACTCTTCTTTCGAAGTGTATAGAG-AGCAGTGTGTGATACGTCGCCTCAAACG 127
Benavente  C--TCTCTACTCTTCTTTCGAAGTGTATAGAG-AGCAGTGTGTGATACGTCGCCTCAAACG 127
CHT-3      CGCTCTCTACTCTTCTTTCGAAGTGTATAGAGCAGCAGTGTGTGATACGTCGCCTCAAACG 239
SQG        CGCTCTCTACTCTTCTTTCGAAGTGTATAGAGCAGCAGTGTGTGATACGTCGCCTCAAACG 239
TaifKSA    CGCTCTCTACTCTTCTTTCGAAGTGTATAGAGCAGCAGTGTGTGATACGTCGCCTCAAACG 220
                * *****

Tt2        ATTAGCAAGAAAAAGACGAATTCGTTTCGCTAGCTGGCGCGCGCGCTTACCGCTTGGAG 187
V.F.       ATTAGCAAGAAAAAGACGAATTCGTTTCGCTAGCTGGCGCGCGCGCTTACCGCTTGGAG 187
Benavente  ATTAGCAAGAAAAAGACGAATTCGTTTCGCTAGCTGGCGCGCGCGCTTACCGCTTGGAG 187
CHT-3      ATTAGCAAGAAAAAGACGAATTCGTTTCGCTAGCTGGCGCGCGCGCTTACCGCTTGGAG 299
SQG        ATTAGCAAGAAAAAGACGAATTCGTTTCGCTAGCTGGCGCGCGCGCTTACCGCTTGGAG 299
TaifKSA    ATTAGCAAGAAAAAGACGAATTCGTTTCGCTAGCTGGCGCGCGCGCTTACCGCTTGGAG 280
                *****

Tt2        AGTACTCGCTCGCGCGAGTACTTCCGATCGTTCTGCGTTCGAGTCCCGGAGCTTTTCTCGAC 247
V.F.       AGTACTCGCTCGCGCGAGTACTTCCGATCGTTCTGCGTTCGAGTCCCGGAGCTTTTCTCGAC 247
Benavente  AGTACTCGCTCGCGCGAGTACTTCCGATCGTTCTGCGTTCGAGTCCCGGAGCTTTTCTCGAC 247
CHT-3      AGTACTCGCTCGCGCGAGTACTTCCGATCGTTCTGCGTTCGAGTCCCGGAGCTTTTCTCGAC 359
SQG        AGTACTCGCTCGCGCGAGTACTTCCGATCGTTCTGCGTTCGAGTCCCGGAGCTTTTCTCGAC 359
TaifKSA    AGTACTCGCTCGCGCGAGTACTTCCGATCGTTCTGCGTTCGAGTCCCGGAGCTTTTCTCGAC 340
                *****

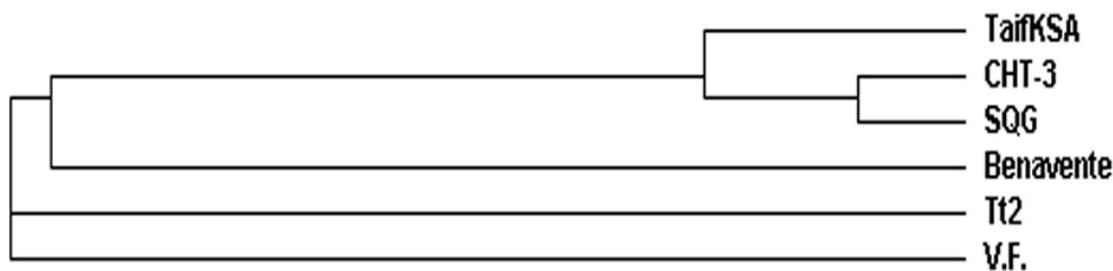
Tt2        TCGTCGAGCAGCGGACCGACGCTTAGCACACGATCAGGCTCGTCCATGCATCGGTCATTG 307
V.F.       TCGTCGAGCAGCGGACCGACGCTTAGCACACGATCAGGCTCCTCCATGCATCGGTCATTG 307
Benavente  TCGTCGAGCAGCGGACCGACGCTTAGCACACGATCAGGCTCGTCCATGCATCGGTCATTG 307
CHT-3      TCGTCGAGCAGCGGACCGACGCTTAGCACACGATCAGGCTCGTCCATGCATCGGTCATTG 419
SQG        TCGTCGAGCAGCGGACCGACGCTTAGCACACGATCAGGCTCGTCCATGCATCGGTCATTG 419
TaifKSA    TCGTCGAGCAGCGGACCGACGCTTAGCACACGATCAGGCTCGTCCATGCATCGGTCATTG 400
                *****

Tt2        AACGCGCGCGCGTGCACTTTTACACGTGGGGCTAGCTCGAA-TTTTTGCTGAACGA-TCT 365
V.F.       AACGCGCGCGCGTGCACTTTTACACGTGGGGCTAGCTCGAA-TTTTTGCTGAACGAGTCT 367
Benavente  AACGCGCGCGCGTGCACTTTTACACGTGGGGCTAGCTCGAA-TTTTTGCTGAACGAGTCT 366
CHT-3      AACGCGCGCGCGTGCACTTTTAC--ATG--GCTAGCTCGAA-TTTTTGCTGAACGAGTCT 474
SQG        AACGCGCGCGCGTGCACTTTTACGCATG--GCTAGCTCGAA-TTTTTGCTGAACGAGTCT 476
TaifKSA    AACGCGCGCGCGTGCACTTTTAC--ATG--GCTAGCTCGAA-TTTTTGCTGAACGAGTCT 455
                ***** ** *****

Tt2        TTTTCT-CGAT----- 376
V.F.       TTTTCTTCGAT----- 379
Benavente  TTTTCT-CGAT----- 377
CHT-3      TTTTCTTCGATCGACGACCTCAGAGCAGGCGAGACCA 512
SQG        TTTTCTTCGATCGACGACCTCAGAGCAGGCGAGACCA 514
TaifKSA    TTTTCTTCGATCGACGACCTCAGAGCAGGCAAGAC-- 491
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**Figure 2.** Multiple alignment of *T. turkistanica* TaifKSA strain sequence with the highest hits in the gene bank.



**Figure 3.** Cladogram of *T. turkestanica* TaifKSA strain and other five strains.

**Table 1.** The biological activities of two strains of *T. turkestanica*; one of them inhabits Taif (TaifKSA) and the second is a commercial strain (SQG).

Biological activity	<i>T. turkestanica</i> TaifKSA mean $\pm$ SD	<i>T. turkestanica</i> SQG mean $\pm$ SD
Developmental time	10.2 $\pm$ 0.6 <sup>a</sup>	9.7 $\pm$ 0.83 <sup>a</sup>
Female longevity	4.1 $\pm$ 0.92 <sup>a</sup>	3.7 $\pm$ 1.23 <sup>a</sup>
Parasitism capacity/female)	41.8 $\pm$ 6.6 <sup>a</sup>	38.55 $\pm$ 8.20 <sup>a</sup>
Emergency (%)	92.6 $\pm$ 7.8 <sup>a</sup>	95.3 $\pm$ 9.24 <sup>a</sup>

Means followed by the same letter on the same row are not significantly different ( $P \leq 0.05$ ).

production often decreases gradually till the end of the female life and the sex ratio is accordingly decreased (Cook, 1993). The previous authors recorded also that the female produces 55.6 eggs. Since this parasitoid is gregarious, no discrepancy between our results and that of the previous authors were found. Hansen (2000) recorded developmental time of 9 days for *T. turkestanica* at 25 to 26°C and this is in agreement with our observation. The success of this biocontrol agent relies not only on the time and the amount of natural enemies released, but also on their quality (emergence, longevity, fecundity and searching capacity) (Bigler, 1994; Dutton et al., 1996).

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