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The characterisation and taxonomic utility of ITS2 in *Tenthredopsis* Costa, 1859 (Tenthredinidae: Hymenoptera) with some new records from Turkey

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ABSTRACT

The taxonomic value of ITS2 has not been well established in insects and the data on its secondary structural properties is limited to a few studies. Here, we present the first data on the secondary structural properties of ITS2 in sawflies and test its utility as a molecular identifier in *Tenthredopsis* (Tenthredinidae: Symphyta). The ITS2 regions were sequenced from 67 specimens representing 14 species and six morphotypes of *Tenthredopsis* and their secondary structure were predicted and characterised. A sequence-structural alignment dataset of ITS2 was analysed by application of maximum likelihood method to see relationship among the specimens. Here, a relaxed approach has been adapted to identify and to elucidate species boundaries by considering the presence of CBCs, hemi-CBCs and unique length size of ITS2 in combination with morphology. The predicted secondary structure of ITS2 was extremely branched in contrast to common core structure found in many eukaryotes. A likely protein binding side was detected in Helix III and suggested to be a synapomorphy for Tenthredinidae. Eighteen species were identified under the relaxed approach applied here, of which *Tenthredopsis lactiflua* (Klug, 1817), *Tenthredopsis ornata* (Serville, 1823), *Tenthredopsis stigma* (Fabricius, 1798) and *Tenthredopsis coquebertii* (Klug, 1817) are new records for Turkey. The structural properties and length size of ITS2 prove useful in determination of species boundaries of closely related species. However, the full account on its taxonomic utility requires more empirical evidence in Hymenoptera.

1. Introduction

The internal transcribed spacer 2 (ITS2) of ribosomal RNA is an essential segment in molecular processes of ribosome maturation by promoting cleavage domains as well as by providing binding sites for nucleolar proteins (Hadjiolova et al., 1994; Lalev and Nazar, 1999). Despite the high mutation rate and common insertions and deletions (indel), the ITS2 forms a highly conserved secondary structure, because of its function in ribosome biogenesis, usually with four helices in eukaryotes (Coleman, 2003; Schultz et al., 2005). ITS2 has increasingly been used in the last decade as a molecular tool for defining

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species boundaries by assessing the structural characteristics, compensatory base changes (CBCs, nucleotide changes at both sides of the paired bases) and length polymorphism (Coleman, 2009; Keller et al., 2010; Wolf et al., 2013). CBCs have been reported to be very informative in species delimitation and a positive correlation has even been established between presence of a CBC and sexual incompatibility, referring to the "biological species" (Müller et al., 2007; Coleman, 2009; Ruhl et al., 2010). The hemi-CBCs (change on only one side of the nucleotide pair, but still preserving pairing) have also been suggested to be useful at population and species level (Torres-Suárez, 2014) but their taxonomic significance is not well established yet.

The utility of ITS2 as a molecular identifier has rarely been tested in insect taxonomy. In insects, only 21,526 ITS2 sequences from 4,386 species have been deposited in GenBank (November, 2015) and this figure is approximately nine times lower than those of Fungi both in sequence and species number. The few applications of ITS2 in insects may be attributed to difficulties related with secondary structure prediction and alignment problems (Wiemers et al., 2009). However, recent advances in these areas lead to the evaluation of even more distantly related taxa and its frequent use in insect taxonomy (Coleman, 2003; Wiemers et al., 2009).

The genus *Tenthredopsis* Costa, 1859 is a member of the largest sawfly family Tenthredinidae and includes almost 60 valid species mostly distributed across the Palaearctic (Taeger et al., 2010). This genus can be distinguished from other genera by the medially undivided first abdominal tergite (Benson, 1952). The species of *Tenthredopsis* are relatively large and brightly coloured species, however, their identification can be unreliable when only colour characters are used. Presence of extreme colour variation in some species may result in misidentification in some cases (Benson, 1968; Blank and Ritzau, 1998; Haris and Gyurkovics, 2014).

Here, the secondary structure properties of ITS2 as a molecular identifier in *Tenthredopsis* (Tenthredinidae: Symphyta) are tested for the first time. We have first sequenced and characterised the predicted secondary structure of ITS2 in 67 specimens of *Tenthredopsis* representing 14 species and six morphotypes which could not be assigned to any species using available identification keys. Second, we have performed phylogenetic analyses based on the structural alignment data to detect likely taxa (species) within 67 specimens. We have adapted a relaxed approach by considering the presence of CBCs, hemi-CBCs and unique length size of ITS2 as a complementary tool to identify species and to elucidate the species boundaries in combination with morphology.

2. Materials and methods

2.1. Samples analysed

A total of 67 ethanol preserved specimens were sorted to species using available identification keys in combination (Benson, 1968; Blank and Ritzau, 1998; Haris and Gyurkovics, 2014; Zhelochovtsev, 1988). Some of the specimens under investigation could not be assigned to any species but constituted six more or less distinct morphotypes and labelled as "*T.* sp. 1 - *T.* sp. 6" hereafter (Table 1). For the different treatment of *Tenthredopsis annuligera* and *Tenthredopsis floricola* by different authors, we followed Benson's concept to our best judgement at present. Voucher specimens were mostly obtained from the Entomological Collection of Cumhuriyet University, Sivas (ECCUS), where previously collected material, was kept in 99% ethanol at −20 °C. Some of the specimens were provided by Senckenberg German Entomological Institute (DEI, Müncheberg, Germany) (Table 1). The ITS2 sequences of *Macrophya* sp. (from this study) and *Empria minuta* (from GenBank; HQ412769) were used as outgroups.

2.2. Generation of sequence data

Whole genomic DNA was extracted from hind leg of the specimens by DNeasy tissue kit (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. The 5.8S-ITS2-28S region was amplified using the following primers: CAS5p8sFc (5′-ATGAACATCGACATTTCGAACGCACAT-3′) and CAS28sB1d (5′-TTCTTTTCCTCCGCTTAGTAATATGCTTAA-3′) (Ji et al., 2003). Amplification was carried out in 50-μl volumes containing 0.5 U of Taq polymerase, 5 μl of 10× reaction buffer (100 mMTris–HCl, pH 8.8, 500 mM KCl and 0.8% Nonidet P-40), 10 pmol of each of the primers, 0.2 mM of each of the four dNTPs, 1.5 mM $MgCl_2$ and 1 μl of DNA template (50–100 ng). PCR cycle conditions were: 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 51.3 °C for 30 s and 72 °C for 1:30 min and finally 72 °C for 5 min. Sequencing reactions were carried out in both directions using the same primers with the PCR reactions. The forward and reverse nucleotide sequences were assembled, edited and aligned by eye using CodonCode Aligner 3.5.6 (CodonCode Corporation, Centerville, MA, USA). Sequences obtained in this study were deposited in the GenBank database with the accession numbers of KU197182-KU197249 (Table 1).

2.3. Inference of secondary structure

For prediction of secondary structures, ITS2 sequences were delimited from the alignment between the 5.8S and 28S proximal stem motifs with the HMM-based annotation tool present at the ITS2 database V (Ankenbrand et al., 2015). E-value < 0.01 and metazoan HMMs with ITS2 minimum size >150 nt were used to define the borders of ITS2. Delimited sequences were submitted to the RNA folding program Mfold Server (Zuker, 2003) to determine putative secondary structures using the following parameters: linear sequence, RNA version 2.3 energy rules, 25 °C. The structures and sequences were synchronously aligned by 4SALE (Seibel et al., 2006) using a specified 12 × 12 scoring matrix (Wolf et al., 2014) in locally