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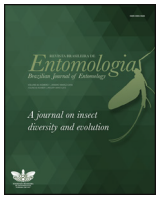

First report on the molecular characterization and phylogenetic analysis of *Fannia* sp. (Diptera: Fanniidae) identified in Türkiye

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ABSTRACT

This study provides the first data on the molecular characterization and phylogenetic analysis of *Fannia* sp. specimens collected from livestock farms in Kırşehir province, Türkiye. Two molecular markers, the mitochondrial DNA *COI* gene and the partial region of the internal transcribed spacer 2 (ITS2) gene, were used for the molecular characterization of the specimens. All *COI* sequences were identical, representing a single haplotype. Similarly, all ITS2 sequences were identical, representing a single genotype. The BLASTn analysis of the Turkish *Fannia COI* haplotype showed 100% similarity to *Fannia canicularis* haplotypes from Portugal, Spain, China, Finland, India, South Korea, Poland, and Canada, and to *Fannia subpubescens* Collin, 1958 from Finland, and *Fannia* sp. from Canada, suggesting these are likely *F. canicularis*. The COI barcode region successfully separated the *Fannia* species into monophyletic clusters in ML and BI analysis. Besides, the BLASTn analysis of the Turkish *Fannia* ITS2 genotype revealed lower similarity (64.2% - 88.4%) to ITS2 sequences from other *Fannia* species. These findings suggest that the mitochondrial *COI* barcode region is a reliable marker for differentiating the *Fannia* species. Further studies are needed to elucidate the genetic diversity and epidemiology of these flies in Türkiye.

Introduction

The Fanniidae are a small family of Diptera distributed across all zoogeographical regions (Durango-Manrique et al., 2024). They inhabit both forested environments and synanthropic habitats (Dominguez, 2023). Some species are drawn to decomposing matter, living vertebrates, carrion, and human remains, making them of great importance in forensic, medical, and veterinary sciences (Aballay et al., 2012; Ziaei Hezarjaribi et al., 2014; Grzywacz et al., 2017). Initially classified as a subfamily of Muscidae (i.e., Fanniinae) in the 1960s based on morphology, the Fanniidae were subsequently recognized as a distinct family within Muscoidea by most specialists (Durango-Manrique et al., 2024). The *Fannia* Robineau-Desvoidy, 1830, is the most widespread genus of Fanniidae with more than 250 species globally and over 150 species in the Palearctic region (Carvalho et al., 2003; Kang et al., 2023; Durango-Manrique et al., 2024). Identification of male fanniids relies primarily on characteristic features such as body color, leg morphology, and genital structures (Rozkošný et al., 1997). In contrast, identification of females and immature stages, often collected from carrion and decaying material, is challenging or not always feasible (Durango-Manrique et al., 2024).

DNA-based molecular identification techniques have emerged as a complementary approach for species identification. These techniques are also advantageous for the identification of old or damaged samples and immature life stages (Fuentes-López et al., 2020). Approximately 650-bp long fragment, which has a greater than 97% species specificity, from the 5' end of the mitochondrial cytochrome c oxidase subunit I (COI) gene was initially proposed as a universal DNA barcode for metazoans (Hebert et al., 2003). However, limitations in discriminating certain taxa of medico-veterinary importance using the *COI* barcode have led to the investigation of alternative genetic markers, including cytochrome b (cytb), internal transcribed spacer 2 (ITS2), and EF1α (Grzywacz et al., 2017).

The presence and distribution of *Fannia* species. have been documented from several regions of Türkiye (Yabas, 1984; Civelek & Tezcan, 2005; Aydemir Çil et al., 2016; Durmaz, 2022;). In addition, Aydenizoz and Gokpınar (2020) reported a case of urinary myiasis in an 8-year-old boy caused by *F. canicularis*. However, no studies have yet explored the genetic characterization of *Fannia* species in Türkiye.

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This study aimed to determine the molecular characterization and phylogenetic relationships of native *Fannia* specimens collected from the Central Anatolia region of Türkiye.

Materials and methods

Sampling and morphological identification

Fannia flies were collected from livestock farms in Kırşehir province in August 2023 using sweep nets. Following collection, the flies were anesthetized with diethyl ether in a disposable plastic bag, put into sterile tubes with 70% ethanol and stored at −20 °C until morphological identification. Fly specimens were initially identified to genus level using a stereomicroscope (Leica EZ4 W, Leica Microsystems, Germany) and established taxonomic keys (Rozkošný et al., 1997; Barták et al., 2016). To examine the genital sclerites, the abdomen was removed, soaked in a 10% potassium hydroxide (KOH) and 5% hydrogen peroxide (H_2O_2) solution, and dissected (Porto et al., 2016).

DNA extraction and PCR amplification

Following morphological identification, two to three legs were removed from each specimen using sterile lancets and placed in microcentrifuge tubes for DNA extraction. The leg samples were individually ground in a microcentrifuge tube under liquid nitrogen using a mortar and pestle. Genomic DNA was extracted using a commercial kit (PureLink Genomic DNA Mini Kit, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Extracted gDNA was eluted in 50 µl of elution buffer and stored at − 20 °C. The concentration of gDNA isolates was determined by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, USA) prior to PCR amplification. Two molecular markers were amplified independently for each sample: a partial fragment of the *COI* gene was amplified with the universal primers (LCO-1490/HCO-2198) described by Folmer et al. (1994) with the annealing temperature set to 50 °C, and the nuclear ITS2 region was amplified with the primers ITS2-F2814 and ITS2-R3295 (Song et al., 2008). Thermal cycling conditions were as follows: initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. Approximately 20 ng of gDNA was used per reaction. PCR products were electrophoresed on 1.5% agarose gel and visualized using a gel documentation system. Amplicons from positive samples were purified using GeneJet Gel Extraction Kit (Thermo Scientific, USA) according to manufacturer's instructions, and sequenced in both directions with amplification primers (BMLabosis, Ankara, Türkiye). All gDNA isolates were screened for the presence of the endosymbiotic bacterium *Wolbachia* with the wsp 81F and wsp 691R primers (Braig et al., 1998).

Sequence and phylogenetic analysis

Raw sequence reads were assessed for quality, and primer sequences were trimmed. The resulting forward and reverse sequences were assembled to create a final consensus sequence using the De Novo Assemble plugin in Geneious 2020.0.3 software (www.geneious.com). The final sequences were compared with reference sequences in the NCBI database by BLAST analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A dataset of genetically related sequences from the GenBank database was then generated for phylogenetic analyses. All sequences were aligned using the ClustalW algorithm in MEGA X (Kumar et al., 2018).

The phylogenetic analyses of the identified haplotype(s) of fanniid flies were performed by the Maximum Likelihood (ML) analyses in MEGA X. In addition, Bayesian inference (BI) analysis of the data set was performed using the Beast v1.10.4 (Suchard et al., 2018). Markov Chain Monte Carlo simulations were run simultaneously for 10.000.000 generations., with sampling every 100 generations for the posterior probability calculations in BI. Before constructing a majority consensus tree, 25% of the initial trees in each run were discarded as burn-in with Treeannotator in Beast v1.10.4. Trees were viewed and edited by Figtree v1.3.1 (Rambaut, 2009). The best DNA-substitution model for ML and BI analyses was selected using the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) algorithms, with jModeltest v.0.1.1 (Posada, 2008). Haplotype analyses, nucleotide composition, and genetic diversity indices were calculated using DnaSP v.5.1. (Librado and Rozas, 2009).

Results

Morphological analysis identified four collected specimens as male *Fannia* sp. (Fig. 1). The COI (648 bp) and ITS2 (366 bp) barcode regions were successfully amplified in the gDNA isolates obtained from the samples. No insertions or deletions were detected in any sequences. *Wolbachia* was not detected in any of the samples. Sequence analysis revealed a single *COI* haplotype. This haplotype exhibited a strong AT bias, with a mean GC content of 30.7%. BLASTn analysis showed 100% similarity to *F. canicularis* haplotypes reported from Portugal, Spain, China, Finland, India, South Korea, Poland, and Canada, as well as to *Fannia subpubescens* Collin, 1958 haplotype from Finland, and *Fannia* sp. haplotypes from Canada. The final *COI* dataset, including GenBank sequences from various global localities, comprised 18 *Fannia* sp. sequences and two *Musca domestica* L., 1958 sequences. Haplotype and nucleotide diversities for the dataset were 0.84 and 0.08, respectively. A total of 147 variable sites were identified, representing 9 distinct haplotypes. Phylogenetic relationships based on the *COI* region are presented in a phylogenetic tree (Fig. 2), constructed using Maximum Likelihood (ML) analysis with the GTR+G nucleotide substitution model. Bayesian Inference (BI) analysis, also using the GTR+G model, yielded a tree topology similar to that of the ML tree; therefore, posterior probabilities and bootstrap values are displayed on the ML tree (Fig. 2). The phylogenetic tree demonstrates clear separation of *Fannia* species, with the included sequences resolved into three major clades (Fig. 2). All sequences were classified into species-specific clades with strong bootstrap support. The newly identified *Fannia* sp. Türkiye haplotype clustered in a monophyletic clade with published *F. canicularis* haplotypes from different countries with high bootstrap value. In addition, the *M. domestica* isolates are clearly separated from the *Fannia* sp. isolates in the dataset.

Analysis of the ITS2 gene sequences from the four isolates revealed identical sequences, indicating a single genotype. In contrast to the *COI* region, the ITS2 region exhibited fewer variable sites (n=78). BLASTn analysis showed various levels of similarity to ITS2 sequences from other *Fannia* species (64,2%-88,4%). The final ITS2 dataset consisted of 15 *Fannia* sp. sequences and two *M. domestica* sequences from different localities around the world. The nucleotide diversity of *Fannia* species in the entire dataset was 0.14. The tree was constructed using Maximum Likelihood (ML) analysis with the GTR+G+I nucleotide substitution model (Fig. 3). While T92 + G was identified as the best fitted model for BI analysis, TN93 + G was used instead because BEAST software does not support T92 + G; TN93 + G was selected as the most comparable alternative model. ML and BI analyses yielded slightly different tree topologies. In the ML tree, the *Fannia* sp. Türkiye isolate clustered as an outer branch to *F. scalaris* isolates from China, with low bootstrap support (45%). In contrast, the BI tree placed the *Fannia* sp. Türkiye isolate as an outer branch to a genogroup containing *F. scalaris* isolates from China and

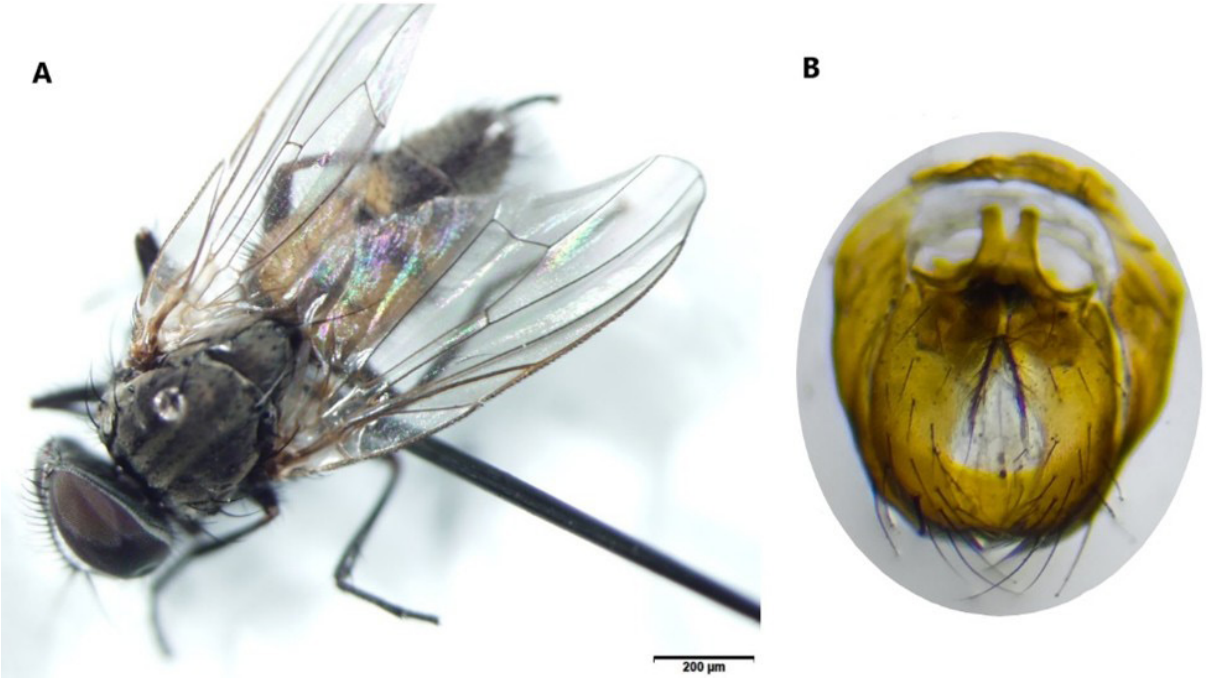

Figure 1 *Fannia* sp., male. (A) Dorsal view, (B) Terminalia.

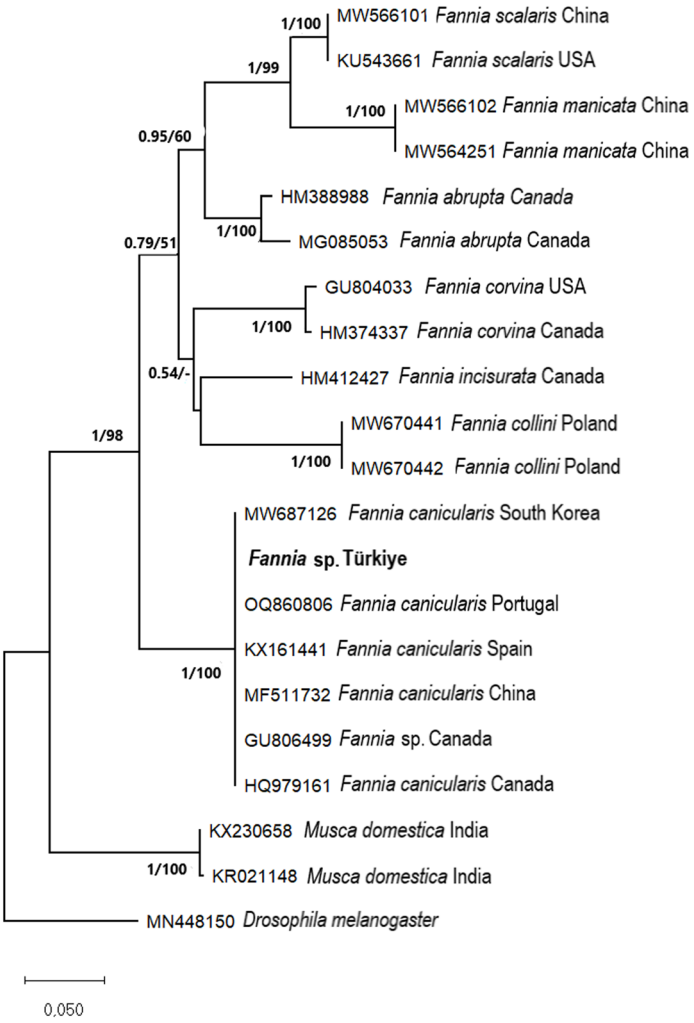

Figure 2 Phylogenetic relationships of *Fannia* sp. haplotype and known haplotypes previously reported from different countries. The tree was constructed using Maximum-Likelihood analyses of *COI* sequences. The isolates are displayed with GenBank accession numbers and country. Nodal supports presented above the line indicate Bayesian posterior probability and ML bootstrap (1000 replicates), respectively. The *Drosphila melanogaster* genotype is used as an outgroup.

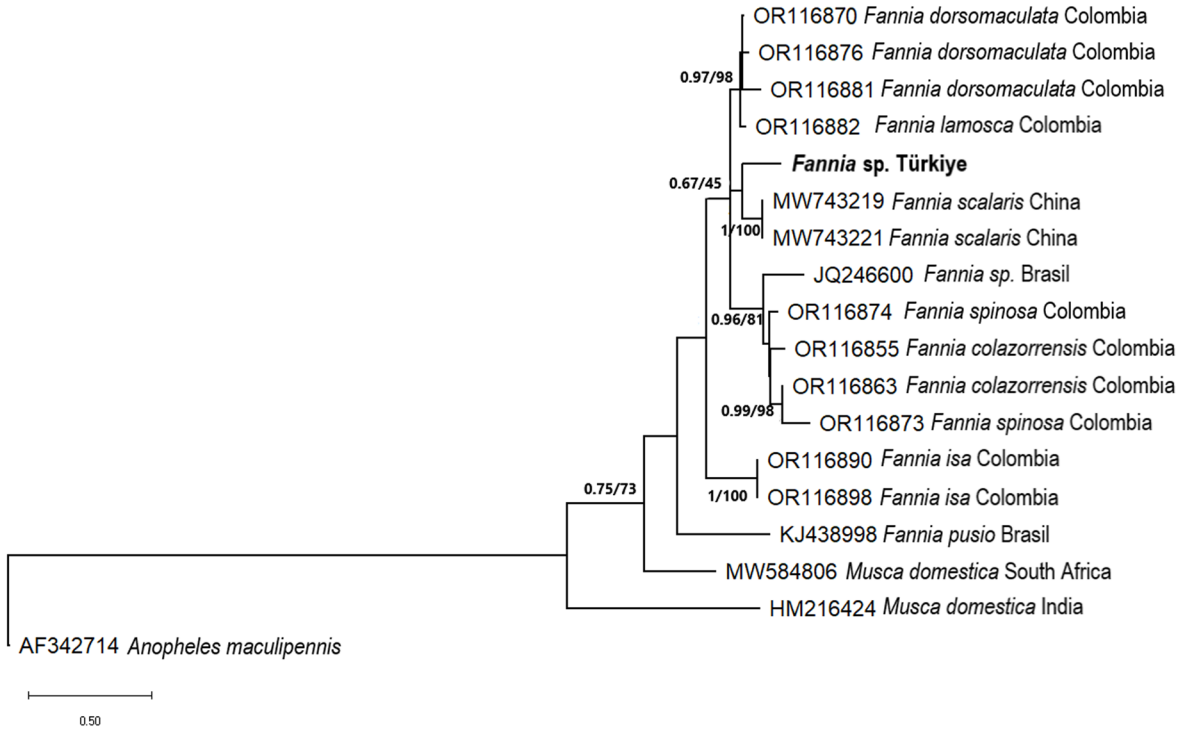

Figure 3 Phylogenetic relationships of *Fannia* sp. genotype and known genotypes previously reported from different countries. The tree was constructed using Maximum-Likelihood analyses of ITS2 sequences. The isolates are displayed with GenBank accession numbers and country. Nodal supports presented above the line indicate Bayesian posterior probability and ML bootstrap (1000 replicates), respectively. The *Anopheles maculipennis* genotype is used as an outgroup.

F. isa, *F. lamosca*, and *F. dorsomaculata* isolates from Colombia, with a posterior probability of 0.66. We also tested the entire ITS2 dataset using Neighbor-Joining (NJ) analysis with Kimura's two-parameter (K2P) model, obtaining results similar to those from the ML analysis.

Discussion

Due to their biology and ecology, several species of Diptera have medical, veterinary, and forensic importance, making them the subject of diverse research across multiple fields. Most of these studies aim to characterize species taxonomically using morphological and molecular features. Although there are a few studies reporting the presence of *Fannia* sp. in several regions of Türkiye, no data are available on the genotypic characterization of these flies. This study provides the first analysis of *COI* and ITS2 sequences from *Fannia* sp. identified in Türkiye. Despite the abundance of *COI* barcode sequences for *Fannia* in GenBank, few ITS2 sequences are available, with no existing sequences for *F. canicularis*. The *COI* barcode region successfully separated the *Fannia* species into monophyletic clusters in ML and BI analysis. The phylogenetic analyses conducted using this gene region have shown that ITS2 was unable to clearly differentiate the *Fannia* species. This discrepancy may be attributed to factors such as differing evolutionary rates between the genes and the limited number of ITS2 sequences in the database. Similarly, Durango-Manrique et al. (2024) found limited discriminatory power for both *COI* and ITS2 in a study of 10 Colombian *Fannia* species, successfully differentiating six species but encountering conflict within the *F. colazorrensis*|*F. dodgei* and *F. laclara*|*F. aburrae* pairs. Song et al. (2008) suggested that the ITS2 gene region can be used to identify necrophagous flies at the species level. However, they noted that different geographical populations of some species, as well as highly homologous species, pose challenges in distinguishing between them. Same researchers reported that the ITS2 sequences were able to differentiate the Sarcophaga at the subgenus level, revealing that the congeneric species clustered in a clade; however, this clustering was not observed in the genus *Lucilia* and the genus *Calliphora*. Marinho et al. (2011) suggested that the ITS2 gene region is informative for phylogenetic analysis of rapidly evolving groups, such as Calyptrate, at both the specific and generic levels (Marinho et al., 2011). In this context, the use of ITS2 as a molecular marker for phylogenetic analysis will continue to be discussed.

Studies have shown that *Wolbachia* infections can limit the effectiveness of mtDNA barcoding (Whitworth et al., 2007; Klopfstein et al., 2016). In this study, all specimens exhibited identical mtDNA COI sequences, indicating a single haplotype, but *Wolbachia* was not detected. Grzywacz et al. (2017) reported similar results for *F. aequilineata*, *F. manicata, F. monilis*, and *F. scalaris*.

In conclusion, this study provides the first genetic characterization of *Fannia* sp. specimens identified in Türkiye, enhancing our understanding of the genetic diversity of Fanniidae Further studies are needed to elucidate the genetic diversity and epidemiology of these flies in Türkiye.

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Conflicts of interest

The author declares no conflicts of interest.

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