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RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

Morphological and molecular diagnosis of the pine processionary moth in Marmara University Göztepe Campus[1]

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Abstract
Pine processionary moth are known as oligophagous forest pests, usually found in cocoons on *Pinus* trees. While these moths are found in the south of Europe and the north of Africa worldwide, in Turkey they are found in the Mediterranean, Aegean, Black Sea and Marmara regions. *Thaumetopoea pityocampa* (Den. & Schiff.) and *Thaumetopoea wilkinsoni* (Tams) species have been observed in the coastal regions of Turkey. Identification of the species is an important step in the development of biological control strategies. The starting point of this study is the desire to control the pine processionary moth in Marmara University Göztepe Campus. However, since both of these species are found in Turkey, especially in the Marmara Region, the main aim of this study is to determine which species the pine processionary moth in the campus belong to. Morphological and molecular methods were used. As a result of the study, based on both morphology and molecular data, it was determined that the species found on the campus was *Thaumetopoea wilkinsoni*.
Keywords: Marmara University, Molecular Systematics, Morphology, Pine Processionary Moth, *Thaumetopoea wilkinsoni*

I. INTRODUCTION

The genus *Thaumetopoea* belongs to the subfamily Thaumetopoeinae (Lepidoptera: Notodontidae) [1]. Pine processionary moths have four stages are observed in the life cycle of pine processionary insects: Egg, larva, pupa and adult. After emerging from their pupae, pine processionary moths mate approximately one week later, and eggs are laid on the leaves of the pine tree a few hours following copulating [2]. While the egg laying process occurs at the end of summer, the eggs hatch approximately 25 days later, at the beginning of autumn [3]-[5]. While pine processionary larvae are 1-2 mm long when they hatch, they can reach 3-4 cm in length at the end of the larval stage [2]. Under normal conditions, the larval stage is the longest phase in the life cycle of the pine processionary moth. The pine processionary larva molts its skin four times and completes its larval stage in five stages [5]. When the larvae hatch, they are white and covered with fine hairs. Among larvae at the same stage, female larvae are larger than male larvae [6].

After spending the winter in the larval stage, pine processionary moths larvae begin to burrow underground in the morning hours in the spring. Pupae spend the summer months underground. Oval cocoons of chestnut color, 20-25 mm in length and 8-10 mm in width are formed [2]. Generally, female pupae are 4 mm longer and 1.5–2 mm thicker than male pupae [5]. The pupal stage, which takes place underground, can last between 1.5 and 7 months [7].

The adult, which completes its development at the end of the pupal period, emerges from the soil in the afternoon at the beginning of the summer. Adult pine processionary moths have bipectinate type antennas and a thin pectinate thorax covered with gray hairs. The forewings are grayish-white, the edges and nerves of the wing are darker, and there are three black transverse bands on the wings. The hind wings are white with gray corners [2]. Pine processionary females are univoltine; they produce offspring only once in their lifetime. Females often die within 24 hours of spawning [3]. Just like the female, the male makes a long flight after mating, falls to the ground and dies when his energy runs out [2], [5].

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The pine processionary moth has adapted to the Mediterranean climate depending on the amount of sunlight and the lowest winter temperature, and accordingly, it plays a very important role in temperature development and dispersion [2]. The most suitable temperature for the development of the pine processionary moth is 20–25°C [8]. Recently, the excessive planting of pine trees in Southern Europe has caused the pine processionary moth to spread over large areas [9]. The increase in average temperature values due to global warming allows the pine processionary larva to live at altitudes where it would not normally survive [4].

The pine processionary moth is found worldwide in the south of Europe and north of Africa [10]. Although there are suitable habitats for the survival of the pine processionary moth throughout the world, their distribution is limited because the female individuals that lay eggs have a weak ability to fly [11]. Countries where pine processionary moth is seen are Germany, Albania, Austria, Bosnia and Herzegovina, Bulgaria, Algeria, Denmark, Morocco, Palestine, France, Croatia, Spain, Israel, Switzerland, Italy, Libya, Lebanon, Hungary, Macedonia, Egypt, Portugal, Romania, Syria, Tunisia, Turkey and Greece [12].

In Turkey, the pine processionary moth has spread to the Mediterranean, Aegean, Marmara and Black Sea regions. Additionally, two species, *T. pityocampa* and *T. wilkinsoni*, are found in Turkey; It has been reported that *T. pityocampa* is distributed in Thrace, Northern Aegean and Western Marmara, and *T. wilkinsoni* is distributed in the Central and Southern Aegean, Eastern Marmara, Mediterranean and Black Sea [13].

The pine processionary moth is an oligophagous insects. Pine processionary moth larvae begin to become harmful when they emerge from their pouches and begin to consume the leaves around them, and the damage they cause in the last larval stage can reach high levels (Devkota and Schmidt, 1990). In a study, it was observed that young Scots pine trees (*Pinus sylvestris* L.) with damaged leaves produced 50% fewer seeds and grew approximately 50% slower than trees without damage [4].

It has been determined that the pine processionary moth also damages *Pinus brutia, P. halepensis, P. nigra, P. pinea, P. radiata, P. strobus, P. sylvestris, Cedrus atlantica, C. deodara* and *C. libani trees* throughout Turkey. Although the pine processionary larvae weaken the host by eating its leaves, short-term damages can be tolerated by the tree because this damage occurs during periods when tree development slows down [2]. Despite this, trees that are constantly under attack by pine processionary larvae enter the new vegetation period with fewer leaves, slowing down their growth rate [14].

The pine processionary moth not only damages forests but also causes allergic reactions in humans and most other homoiterm animals [15]. "Catterpillar dermatitis" occurs when contact with pine processionary larvae occurs; local symptoms of redness, swelling, itching and pain are observed on the skin [16].

When determining the control method, the interaction of the pine processionary moth with biotic and abiotic factors should be examined, and the method to be followed should be prevented from harming these factors [17]. Fighting against the pine processionary moth can be achieved by biological, chemical, mechanical and cultural means [2]. Among the alternatives, biological control is the most effective method, while chemical method is the most dangerous, therefore it has been observed that biological control methods are mostly preferred. Biological control is carried out through species-specific competing populations, the main purpose of which is to reduce the density of the species being combated and make it less harmful [18].

When it comes to biological control, all forms of the pine processionary moth can have enemies. As a result of an experiment, it was observed that the pine processionary moth attack was reduced by 19.3% after the egg parasites were used in biological control [19]. In Europe and Israel, *Ooencyrtus pityocampae* (Mercet) (Hymenoptera: Encrytidae) and *Baryscapus servadeii* (Domenichini, 1965) (Hymenoptera: Eulophidae) have been recorded as primary *T. pityocampa* egg parasitoids [2]. In another study, *O. pityocampae* and *B. servadeii* species were identified as the most common pine processionary parasitoids in Isparta [20].

The continuous presence of the pupa in the soil and the formation of pupae heaps as the larvae move together increases the effectiveness of biological control through the enemies of the pine processionary moth pupae (Battisti et al., 2000). In this context, *Formica rufa* (Hymenoptera: Formicidae) (Red forest ant) was used to combat the pine processionary moth and the results were found to be successful [21].

In addition to biological control, it is aimed to reduce the population density within the scope of biotechnical control: Establishing pheromone traps by synthetically producing secreted pheromones so that adults can find each other is one of these methods [2].

In this study carried out within the scope of the TÜBİTAK 2209/A project, it was aimed to determine the species of pine processionary moth found in Marmara University Göztepe Campus using morphological and molecular methods. The results obtained through the study are intended to contribute to the determination of methods to combat the pine processionary moth.

II. MATERIALS AND METHOD

2.1 Sample Collection from the field

On 02.02.2023, sacs of the pine processionary moth were observed on *P. brutia* species in Marmara University Göztepe Campus (40.987252; 29.053699, 44 m) (Figure 1). Sacs were collected by pruning-shears from three *P. brutia* trees distributed in different areas in this campus and larvae were obtained from the sacs (Figure 2). Then all sacs were brought to the laboratory.

Figure 1. The pine processionary moth pouches seen in Marmara University Göztepe Campus

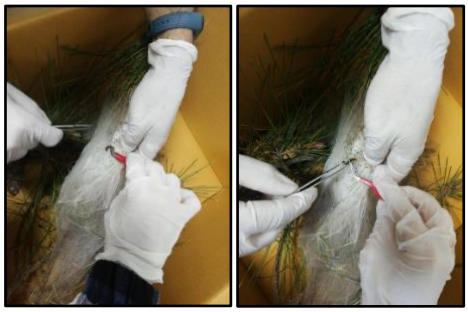

Figure 2. Larvae of the pine processionary moth collected at Marmara University Göztepe Campus

2.2 Species Identification of Specimens According to Morphological Characteristics

Twelve of the larvae collected from three different trees were placed in 90% alcohol (in 2 ml sterilized cryo tubes) and stored at -20°C for molecular species identification. The larvae in the remaining sacs were kept in glass aquariums (with soil underneath) at 25°C and 60% RH humidity conditions and reared under 12:12 hours light:dark photoperiod conditions for morphological identification. Larvae then moved into the soil to enter the pupal stage. Pupated larvae were kept at 25°C, 60% RH humidity and continuous dark conditions. After about 3 months, adults emerging from the pupae were collected and necessary dissection procedures were carried out in the laboratory for species identification.

Although classical systematic studies in insects are based on external body morphology, in some cases this method is not sufficient. For example, it is very difficult or even impossible to make a morphological species identification in sibling species. For this reason, genitalia are an important morphological character used for species identification.

Morphological diagnoses of species belonging to the genus *Thaumetopoea* are usually made according to canthus structure. Adult specimens were killed with ethyl acetate species identifications were made according to the structure of the removed canthus.

2.3 Species Identification of Specimens by Molecular Systematic Methods

DNA isolation was performed from the larvae collected for molecular species determination. DNA isolation was performed manually. DNA quality and quantity were determined with Take3 plate and Cytation3 (Biotek, USA) device. Approximately 810 bp of the cytochrome oxidase I (COI) gene region in mtDNA was amplified by PCR from the samples in 90% ethyl alcohol. Primer pairs given in Table 1 were used to amplify the gene region to be studied. The prepared mixtures were loaded into microplate wells and placed in a thermal cycler. PCR reactions were performed in a Biorad (USA) brand thermal cycler. Amplicons were visualized by agarose gel elctrophoresis and purified by gel recovery kit. The pure amplicon was then sequenced by outsourced sequencing. The sequences obtained were compared with GenBank data by BLAST algorithm and species determinations were performed.

Table 1. Primers used to amplify the gene region to be sequenced

Gene	Series	Source
COI	Jerry(forward)C1-J-2183 5'- CAACATTTATTTTGATTTTTTG G-3' Pat(reverse)TL2N-3014 5'- TCCAATGCACTAATCTGCCAT ATTA-3'	Simon et al., 1994

The head of the larvae in 90% ethyl alcohol was dissected and the samples were taken into 1.5 ml sterile ependorfs. Sterile glass beads (for sample disintegration) and 300 µl KCL buffer were added to the same ependorfs. These samples were then physically lysed in a MagNa Lyser at 5000 rpm for 1 minute. Then 300 µl of chloroform was added to the ependorfs and vortexed for 30 seconds. The ependorfs were then centrifuged at 12000 rpm for 3 minutes at +4°C. After centrifugation, 200 µl of the supernatants of the centrifuged samples were taken and transferred into 1.5 ml sterile ependorfs. Then 120 µl of cold isopropanol was added to the samples. For 30 seconds, the samples were mixed by hand movement. The

samples were then centrifuged at 12000 rpm for 2 minutes at +4°C. The supernatant was gently discarded and 200 µµl of 96% cold ethanol was added to the samples. Centrifugation was again performed at 12000 rpm for 2 minutes at +4°C. The liquid phase was slowly poured off and the tubes were allowed to dry upright to evaporate the ethyl alcohol. Then 100 µl of TE buffer was added and stored at +4°C until NanoDrop processing. DNA quality and quantity were determined on a Take3 plate with Cytation3 (Biotek, USA).

A mitochondrial nuclear gene region (cytochrome oxidase subunit I (COI)) was amplified from the isolated DNA using the following primers and PCR protocol. Prepared mixtures (PCR Mix) and samples were loaded into microplate wells and placed in a thermal cycler. PCR reactions were performed in a Biorad (USA) brand thermal cycler.

PCR protocol for the COI gene region [22]: 1 cycle: 94°C 5 min; 35 cycles: 94°C 45 s, 45°C 30 s, 72°C 1 min; 1 cycle: 72°C, 2 min

The Cytochrome Oxidase I gene region amplified by PCR from the samples was run on 2% agarose gel electrophoresis using non-toxic electrophoresis dye (Safeview etc.) for each sample and the results of the PCR were checked according to the amplicon lengths. The PCR products were then purified using a gel recovery kit. The pure amplicons were then sequenced by the Sanger method by outsourcing.

The raw data obtained by Sanger sequencing were opened with SnapGene 7.0.2 software and analyzed based on base quality scores. Regions of 20-30 nucleotides with low quality scores at the beginning of the sequence were deleted with trim and bases with a quality score (Phred) above 20 were preferred. The two-way reads obtained with Sanger were then merged with NCBI Blast to obtain a consensus sequence. The NCBI BLASTN algorithm was applied to compare the consensus sequence with the reference database of COI gene sequences from various species. The best BLAST result was used to assign a taxonomic identity based on the COI gene sequence. At the same time, the consensus sequence was also searched in the BOLD database using the Identification Engine and the identification report was obtained.

III. RESULTS AND DISCUSSION

As a result of species identification made from adult pine processionary moth taken from three different trees, it was determined that the pine processionary moth found on campus were *Thaumetopoea wilkinsoni* species. Since it is difficult to identify species from the larvae of the species belonging to the genus, species identification was made using adult pine processionary moth. The canthus structure of male pine processionary moth was examined (Figure 3). The studies were compared with the existing literature [23]-[25]. According to the canthus structure, it has been determined that the pine processionary moth detected in Marmara University Göztepe Campus are *Thaumetopoea wilkinsoni* species.

A **B**

Figure 3. Canthus structure: A. *Thaumetopoea pityocampa* [26] B. *Thaumetopoea wilkinsoni* (drawed by authors)

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