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MOLECULAR CONFIRMATION OF *STETHORUS* (*STETHORUS*) *PUSILLUS* (HERBST, 1797) IN NORTHERN SERBIA

IVANA JOVIČIĆ[1*] and SLAVICA MARINKOVIĆ[2]

1 Laboratory of Applied Entomology, Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Belgrade, Zemun, Serbia

2 Department of Plant Pests, Institute for Plant Protection and Environment, Banatska 33, 11080 Belgrade, Zemun, Serbia

E-mail*: ivana.jovicic@pesting.org.rs (corresponding author)

Abstract

Stethorus (*Stethorus*) *pusillus* (Herbst, 1797) (Coleoptera: Coccinellidae), one of the most effective biocontrol agents against harmful mites in horticultural crops, was recorded for the first time in Sremska Mitrovica, northern Serbia, in August 2022. Molecular analyses confirmed the morphological identification. This Holarctic species primarily preys on mites on many cultivated plants. While the specimens in this study were found on peach (*Prunus persica* L.) leaves, the presence of mites was not documented at the time of sampling.

KEYWORDS: ladybirds, fauna, predator, Stethorini tribe

Introduction

The genus *Stethorus* Weise, 1885 (Coleoptera: Coccinellidae) has a cosmopolitan distribution. Species in this genus primarily feed on mites, although occasional predation on aphids and thrips has been reported (Kapur, 1948). Furthermore, reported food sources include tobacco whitefly on cotton (Silva & Bonani, 2008), as well as raisins, aphid honeydew, and peach leaf extrafloral nectar (Pemberton & Vandenberg, 1993).

Stethorus is easily distinguished from other coccinellids by its small body size, typically ranging from 1-1.5 mm in length, mostly black in color, with yellowish-white setae, predominantly yellow legs, and an absence of markings on the pronotum and elytra (Kapur, 1948; Bieńkowski, 2018; Seki & Maruyama, 2025). The morphology of *Stethorus* is highly variable, requiring a rigorous identification process that integrates morphological and molecular analyses (Biddinger *et al.*, 2009; Vidya *et al.*, 2022).

Initially treated as a subgenus of *Scymnus* Kugelann, 1794, *Stethorus* was subsequently elevated to genus status (Kapur, 1948; Seki & Maruyama, 2025). The genus *Stethorus* is divided into two subgenera: *Stethorus* s. str. and *Allostethorus* Iablokoff-Khnzorian, 1972, with approximately 30 and 50 species, respectively (Seki & Maruyama, 2025).

According to the Alciphron database (alciphron.habiprot.org.rs) and Stančić (2013), only one species of this genus, *Stethorus* (*Stethorus*) *pusillus* (Herbst, 1797), has been identified in Serbia to date. The most used synonym is *Stethorus* (*Stethorus*) *punctillum* Weise, 1891 (Seki & Maruyama, 2025).

Materials and Methods

Coccinellids were sampled from a ten-year-old peach orchard in the city of Sremska Mitrovica, which is managed according to an integrated pest management (IPM) system. Specimens were preserved in 70% and 96% ethanol and stored in a refrigerator at 4°C. Coccinellids were observed and photographed using an Olympus SZ 61 stereo microscope (Olympus, Tokyo, Japan) with a maximum magnification of 45× and an integrated camera, and identified using several keys (Kapur, 1948; Bieńkowski, 2018; Seki & Maruyama, 2025).

To verify the morphological identification, a single adult was analyzed using the DNA barcode region of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene (Hebert *et al.*, 2003). Total genomic DNA was extracted using the EZNA Insect DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions. Extracted DNA was stored at -20°C until further use. Amplification of the COI barcode region (658 bp) was performed using the universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). PCR reactions were carried out in a final volume of 25 µL, containing 12.5 µL of 1× PCR Master Mix (Fermentas, Vilnius, Lithuania), 10.5 µL nuclease-free water, 0.5 µL of each primer (10 µM), and 1 µL of DNA. PCR amplifications were conducted in a Biometra T-Personal PCR Thermocycler (Analytik Jena GmbH+Co., Jena, Germany) following the thermal protocol described by Massimino Cocuzza and Cavalieri (2014). Amplification success was confirmed on a 1% agarose gel stained with 0.1% ethidium bromide and visualized under a UV transilluminator (LKB Bromma, Stockholm, Sweden). All PCR amplicons were sequenced in both directions using the same primers by a commercial sequencing service (Macrogen Inc., Seoul, South Korea). The obtained sequence was assembled using FinchTV v.1.4.0 (finchtv.software.informer.com) and aligned with CLUSTAL W integrated in MEGA7 software (Kumar *et al.*, 2016). Prior to analysis, the sequence was translated into amino acids to confirm the absence of stop codons. The obtained sequence was compared with those deposited in the National Center for Biotechnology Information (NCBI) GenBank database (blast.ncbi.nlm.nih.gov) using the BLAST nucleotide search tool.

Specimens and extracted DNA samples were deposited in the collection of the Institute of Pesticides and Environmental Protection in Belgrade (Serbia).

Results

Based on morphological and molecular analyses, the species was identified as *Stethorus* (*Stethorus*) *pusillus* (Herbst, 1797).

Collected material: Three adult females. All individuals were collected by hand from the adaxial side of leaves on a single peach tree in the middle of the canopy. No mites or aphids were found on peach shoots where coccinellids have been collected.

Locality and date: City of Sremska Mitrovica (45°00'04" N, 19°38'12" E), northern Serbia, 25 August 2022 (Fig. 1).



Figure 1. Collection locality of *Stethorus* (*Stethorus*) *pusillus* in Serbia.

Collector: Ivana Jovičić.

Morphology: The body is predominantly black, shortly oval, and moderately convex, approximately 1.3 mm in length (Fig. 2). The head is finely and sparsely punctate, with antennae nearly as long as the width of the clypeus. The pronotum is unevenly punctate. The elytra are dark greyish, moderately long, with sparse, semi-erect yellowish pubescence. The antennae and the apices of the femora, tibiae, and tarsi are brownish to yellowish.

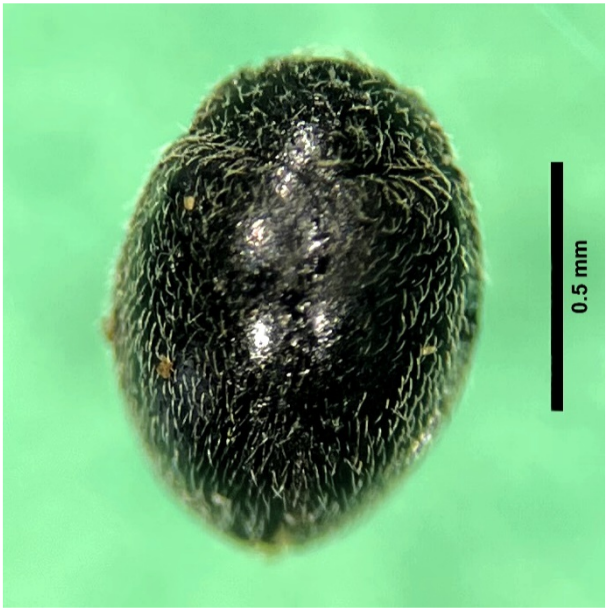


Figure 2. *Stethorus* (*Stethorus*) *pusillus*, female (photo: I. Jovičić).

Taxonomy: Order Coleoptera Linnaeus, 1758; suborder Polyphaga Emery, 1886; family Coccinellidae Latreille, 1807; subfamily Scymninae Mulsant, 1846; tribe Stethorini Dobzhansky, 1924; genus *Stethorus* Weise, 1885; subgenus *Stethorus* Weise, 1885.

Molecular study: The complete barcode region, encoding 219 amino acids of *S.* (*S.*) *pusillus*, was successfully amplified and sequenced. The resulting sequence has been deposited in GenBank under accession number PX688282.

Blast searches of the COI sequence from the Serbian specimen identified a sequence labelled as *S. punctillum* from Germany (GenBank accession number KM448697; Hendrich *et al.*, 2015) as the closest match exhibiting 100% sequence identity. The sequence labelled as *S.* (*S.*) *pusillus* from Finland (GenBank accession number KJ963340; Pentinsaari *et al.*, 2014) showed slightly lower similarity (99.24%). Amino acid translation revealed no substitutions, indicating full conservation of the encoded protein among the analyzed sequences.

Discussion

To date, *S.* (*S.*) *pusillus* has been documented in Serbia in the Autonomous Province of Vojvodina, near the town of Obrenovac, in the Ram-Golubac Sands, in the vicinity of Niš, and in southern Serbia near the border with North Macedonia (Grbić *et al.*, 1989; Injac & Perić, 1989; Stančić, 2013; Alciphron, 2014–2026; Damjanović & Stanković, 2017). In this study, the species was recorded for the first time in Sremska Mitrovica. The nearest previous record of *S.* (*S.*) *pusillus* is from the Zasavica Special Nature Reserve (Damjanović & Stanković, 2017). This coccinellid is considered native to Europe and exhibits a cosmopolitan distribution (Seki & Maruyama, 2025).

Although Biswas *et al.* (2007) reported a sex ratio of approximately 1:1 in *S.* (*S.*) *pusillus*, only female individuals were collected in the present study. Consequently, the male genitalia, whose features are considered critical for accurate identification within the genus *Stethorus* (Kapur, 1948), could not be examined. This was the main reason for conducting a molecular analysis of the collected specimens. Furthermore, adults across all Stethorini species are small, cryptic, and characterized by black bodies and brown or yellow appendages; as a result, misidentifications are frequent (Biddinger *et al.*, 2009). The mitochondrial COI gene has been widely used for the identification of many insects (Hebert *et al.*, 2003), including coccinellids (Vidya *et al.*, 2022). Molecular analyses conducted in this study confirmed the morphological identification of *S.* (*S.*) *pusillus* and supported the synonymy of this species with *S. punctillum*. These results are consistent with previous phylogenetic studies showing that *S.* (*S.*) *pusillus* is nested within the *S. punctillum* clade (Vidya *et al.*, 2022).

While most species within the family Coccinellidae primarily prey on aphids or scale insects, approximately 90 species of the tribe Stethorini, belonging to the genera *Stethorus* Weise, 1885 and *Parastethorus* Pang & Mao, 1975, are specialized mite predators (Biddinger *et al.*, 2009; Seki & Maruyama, 2025). Members of this tribe occur on a wide variety of plants, including numerous cultivated hosts such as pome and stone fruits (Kapur, 1948; Biddinger *et al.*, 2009). The primary prey of the genus *Stethorus* consists of mites from the genera *Eotetranychus* Oudemans, 1931, *Oligonychus* Berlese, 1886, *Panonychus* Yokoyama, 1929, and *Tetranychus* Dufour, 1832 (Biddinger *et al.*, 2009). However, they are occasionally found within aphid colonies, such as those of *Phorodon humuli* (Schrank, 1801) (Kapur, 1948). In the present study, *Stethorus* (*Stethorus*) *pusillus* (Herbst, 1797) was recorded on peach foliage during a period when mites and aphids were absent, suggesting that it may have been feeding on peach leaf extrafloral nectar, a behavior previously noted as a possibility by other authors (Pemberton & Vandenberg, 1993).

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МОЛЕКУЛАРНА ПОТВРДА ПРИСУСТВА *STETHORUS* (*STETHORUS*) *PUSILLUS* (HERBST, 1797) У СЕВЕРНОЈ СРБИЈИ

Ивана Јовичић и Славица Маринковић

Извод

Врста *Stethorus* (*Stethorus*) *pusillus* (Herbst, 1797) (Coleoptera: Coccinellidae), један од најефикаснијих природних савезника повртара и воћара у борби против штетних грињa, нађена је по први пут у Сремској Митровици (северна Србија) 25. августа 2022. године. Морфолошка идентификација врсте је потврђена молекуларним методама. Ова холарктичка врста се примарно храни грињама на многим гајеним биљкама. У нашим истраживањима, врста је забележена на листовима бресквe (*Prunus persica* L.), али присуство грињa није забележено.

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