JOURNAL OF AGRICULTURE

RESEARCH ARTICLE ISSN: 2636-8757

Phylogenetic Insights into *Sitona* Germar, 1817 (Coleoptera: Curculionidae) through DNA Barcoding of the COI Gene

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This study was financially supported by The Scientific and Technological Research Council of Turkey with project number 120O352.

Abstract: Sitona Germar is a large genus in the family Curculionidae, comprising approximately 100 species. However, recent revisions of subgenera or species groups classifications are lacking, making it difficult to compare newly discovered species with previously described ones. In this study, we performed a molecular phylogenetic analysis based on the COI (cytochrome c oxidase subunit I) gene to assess the validity of the traditional groupings within the genus Sitona. A total of 23 species were analyzed, 14 of them were collected in the eastern and southern regions of Türkiye: S. callosus Gyllenhal, S. concavirostris Hochhuth, S. cylindricollis Fåhraeus, S. fairmairei Allard, S. hispidulus (Fabricius), Sitona humeralis Stephens, S. lateralis Gyllenhal, S. lineatus (Linnaeus), S. lividipes Fåhraeus, S. longulus Gyllenhal, S. macularius (Marsham), S. obsoletus (Gmelin), S. puncticollis Stephens and S. sulcifrons Thunberg. In addition, nine species were incorporated from GenBank records: S. ambiguus Gyllenhal, S. brucki Allard, S. discoideus Gyllenhal, S. languidus Gyllenhal, S. striatellus Gyllenhal, S. suturalis Stephens and S. waterhousei Walton. Two outgroup taxa, Coelositona latipennis (Gyllenhal) and C. limosus (Rossi), were also included in the analysis. Phylogenetic reconstruction was performed using the Maximum Parsimony method with 1000 bootstrap replicates, implemented with Geneious Prime (2024.0.7) and the PAUP 4.0a plug-in. The resulting phylogram grouped the analyzed species into four distinct clusters, which was inconsistent with Reitter's previous classification of Sitona. The traditional morphological characters used in the taxonomy of the genus are briefly reviewed, and a new species grouping framework is proposed.

Keywords: Molecular phylogeny, Species identification, Insect systematics, Genetic divergence, Türkiye.

INTRODUCTION

The tribe Sitonini comprises seven genera in the Palaearctic region: Andrion Velázquez de Castro, 2007; Charagmus Schoenherr, 1826; Coelositona González, 1971; Eugnathus Schoenherr, 1834; Schelopius Desbrochers des Loges, 1872; Sitona Germar, 1817; Velazquezia Alonso-Zarazaga and Lyal, 1999. The genus Sitona consists of 115 species, with 109 species distributed throughout the Palaearctic region (Alonso-Zarazaga, et al., 2023) and six species native to North America (Bright, 1994). Sitona larvae primarily feed on roots and root nodules (Scherf 1964), while adults consume the leaves of Fabaceae plants (Velázquez de Castro et al., 2007; Gözüaçık, 2023). Due to this feeding behavior, many Sitona species are considered important agricultural pests, causing considerable losses in crops (Aeschlimann, 1980; Syrett, 1992; Murray, 1996; Cantot, 2001).

The first attempt to classify Sitona species into groups was made by Stierlin (1885), who proposed five groups. However, the first two currently correspond to the genera Charagmus, Andrion, and Coelositona. Later, Reitter (1903) introduced 11 species groups, which were widely accepted for several decades (Porta, 1932; Hustache, 1946; Hoffmann, 1950; Boroumand, 1975; Alonso-Zarazaga, 2002). Over time, some of Reitter's groups were reassigned to other genera within Sitonini, leaving only eight groups currently recognized within Sitona. The morphological characters used by Reitter to define these groups are summarized in Table 2.

Received: 12-09-2025 | Accepted: 02-11-2025

Velázquez de Castro (1997) carried out a detailed morphological study of the genus Sitona, reexamining several of its diagnostic features. One such feature is the presence of a precoxal zone in the prosternum, which consists of a space that may exist between a groove on the anterior part of the pronotum and the proacetabula. While Reitter (1903) merely noted the presence or absence of this trait, Velázquez de Castro (1997) refined this assessment by introducing a quantitative measure, comparing the size of the precoxal zone relative to the body size (p = size of precoxal zone/body size). Velázquez de Castro also identified several new morphological characters, including the shape of the sclerites within the internal sac of the aedeagus.

DNA barcoding has been used more recently for species identification and classification (Hollingsworth et al., 2009; Hollingsworth, 2011; Coissac et al., 2016). Previous studies on Sitona have primarily been part of broader analyses of the superfamily Curculionoidea and have included a small number of Sitona species (Schütte et al., 2013; Stüben et al., 2015; Stüben, 2022; Schütte et al., 2023). The aim of our study is to expand on these efforts by investigating phylogenetic relationships within Sitona, including a larger number of species in genetic analysis and using morphological characters.

MATERIAL and METHODS

Collection of samples

Fourteen species of Sitonini were collected using a sweep net from alfalfa, sainfoin, and wild legume fields in east and south region of Türkiye between April and November 2020–2021. The collected specimens were immediately placed into tubes containing 96% ethanol for preservation. In the laboratory, the specimens were examined under a microscope for identification. After diagnosis, the samples were transferred to 95% ethanol and stored at -20°C until DNA extraction. The specimens are housed in the Entomology Laboratory of the Department of Plant Protection, Faculty of Agriculture, Iğdır University. Detailed information on the collection sites, including geographical coordinates and GenBank accession numbers, is provided below (Table 2).

DNA extraction and PCR amplification

Three legs (approximately 20 mg) from each specimen were dissected for DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen, Cat. No. 69504) following the manufacturer's protocol. DNA integrity was assessed by 1% agarose gel electrophoresis, concentration quantified using a Qubit 2.0 fluorometer with the Qubit dsDNA BR Assay Kit (Invitrogen, Cat. No. Q32851), and purity determined using a NanoDrop spectrophotometer (MaestroNano, Maestrogen). DNA samples were normalized to 10 ng/µL and stored at −20 °C until PCR amplification.

The COI fragment was amplified in a 25 µL reaction containing 2× Reaction Buffer (Thermo Scientific, Cat. No. EP0401), 0.1 mM dNTPs, 0.2 µM of each universal primer LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994), 1 U Taq DNA polymerase (Thermo Scientific, Cat. No. EP0401), 1 mM $MgCl_2$, 20 ng of template DNA, and nuclease-free water. PCR amplification was performed on a SimpliAmp™ thermal cycler (Thermo Scientific) under the following conditions: initial denaturation at 95 °C for 1 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Amplicons were verified on a 2% agarose gel.

***Table 2**. Sampling details used in the present study with GenBank accession numbers*

Code	Species	Collection Site	Coordinates	Accession
107-2	*Sitona humeralis*	Bingöl-Karlıova, Türkiye	39°12′47″N, 40°57′44″E	OP629518
UZ3	*Sitona macularius*	Mardin-Derik-Kuşçu, Türkiye	37°18′27″N, 40°16′11″E	OP629522
105	*Sitona obsoletus*	Bingöl-Sarıçiçek, Türkiye	38°5′39″N, 40°36′26″E	OP629517
137	*Sitona hispidulus*	Ağrı-Patnos-Değirmen, Türkiye	39°10′38″N, 43°2′36″E	OP629519
199	*Sitona callosus*	Ağrı-Eleşkirt-Goncalı, Türkiye	39°51′4″N, 42°51′23″E	OP629520
102-2	*Sitona lineatus*	Bingöl-Genç-Gümüşlü, Türkiye	38°44′48″N, 40°30′40″E	OP629516
1	*Sitona lividipes*	Bingöl-Genç-Gümüşlü, Türkiye	38°44′48″N, 40°30′40″E	OP629509
4	*Sitona lateralis*	Diyarbakır-Çermik-Karataş, Türkiye	38°3′11″N, 39°25′2″E	OP629512
3	*Sitona cylindricollis*	Van-Çaldıran-Avcıbaşı, Türkiye	39°8′19″N, 43°54′34″E	OP629510
5a5	*Sitona concavirostris*	Şanlıurfa-Siverek-Ediz, Türkiye	37°41′6″N, 39°15′8″E	OP629513
6	*Sitona puncticollis*	Muş-Şenoba, Türkiye	38°53′43″N, 41°30′54″E	OP629514
8	*Sitona sulcifrons*	Iğdır-Merkez-Suveren, Türkiye	39°48′38″N, 44°04′ 25″E	OP629515
2	*Sitona longulus*	Van-Muradiye-Kemerköprü, Türkiye	39°2′8″N, 43°45′31″E	OP629510
F1-5	*Sitona fairmairei*	Kahramanmaraş-Ilıca, Türkiye	37°50′23″N, 36°56′03″E	OQ077664
	*+*Coelositona limosus*	Adıyaman-Besni-Akpınar, Türkiye	37°45′45″N, 37°43′31″E	MW520732
	*+*Coelositona latipennis*	Spain	-	KC783802
	**Sitona languidus*	Germany	-	HQ953708
	**Sitona waterhousei*	Germany	-	KM451971
	**Sitona striatellus*	Germany	-	KM442362
	**Sitona ambiguus*	Czechia	-	KC783942
	**Sitona suturalis*	Poland	-	KC784204
	**Sitona brucki*	Morocco	-	KC783883
	**Sitona dicoideus*	Norfolk Island	-	EF118296

*: Data retrieved from GenBank. -: No data available. +: Selected as outgroup.

Sequencing and phylogenetic analysis

PCR products were purified and subjected to bidirectional Sanger sequencing (ABI 3730xl System) at Macrogen Inc. (The Netherlands) using the same primer pair as in the PCR amplification. Raw sequence files were imported into Geneious Prime (2024.0.7), where sequencing quality was assessed, primer-binding regions were trimmed, and forward and reverse reads were aligned to generate consensus sequences. These consensus sequences were deposited in GenBank under the accession numbers listed in Table 2.

For phylogenetic reconstruction, sequences were aligned using the Geneious Aligner with default parameters (cost matrix: 65% similarity; alignment type: global with free end gaps). Maximum Parsimony (MP) analysis was performed in PAUP 4.0a (Swofford, 2003) using a heuristic search with 1000 random addition replicates, holding one tree at each step, and treating gaps as missing data. *Coelositona limosus* and *C. latipennis* were selected as outgroups, consistent with Stüben (2022), which identified Macaronesian species of *Coelositona*, including *C. latipennis*, as the sister group to *Sitona*. Although *C. limosus* has a Mediterranean distribution, it belongs to the same Macaronesian group based on morphological features such as internal sac sclerite structure, the shape of the eighth female sternite, a globose pronotum, and relatively large body size.

RESULTS and DISCUSSION

The extracted DNA yielded concentrations of 50–85 ng/µL (A260/A280 = 1.7–2.1; A260/A230 = 1.9–2.2), suitable for PCR amplification of the barcode region. Sequencing quality scores ranged from 84.2% to 99.7%. After trimming, the aligned sequence length used for phylogenetic analysis was 445 bp.

No stop codons were detected, and BLAST searches confirmed that the obtained sequences corresponded to functional mitochondrial genes. Nucleotide composition varied among species, with thymine (T) being the most abundant (36.05%) and guanine (G) the least (16.83%). *Coelositona limosus* exhibited the highest GC content (35.1%), whereas *Sitona lividipes* had the lowest (29.3%). Overall, sequences displayed a high adenine-thymine (A–T) bias (67.69%), which is typical of insect mitochondrial COI sequences (Crozier & Crozier, 1993; Dowton & Austin, 1995; Whitfield & Cameron, 1998).

Among the 445 sites, 268 were conserved, 177 were variable, and 125 were parsimony informative. Transition substitutions (si = 35) were more frequent than transversions (sv = 33), resulting in a transition/transversion ratio (R = si/sv) of 1.083.

Remarks on the phylogram:

The phylogram shows two unclustered species and a big group of *Sitona* which is, divided in two groups, group A and group B + C (Fig. 2).

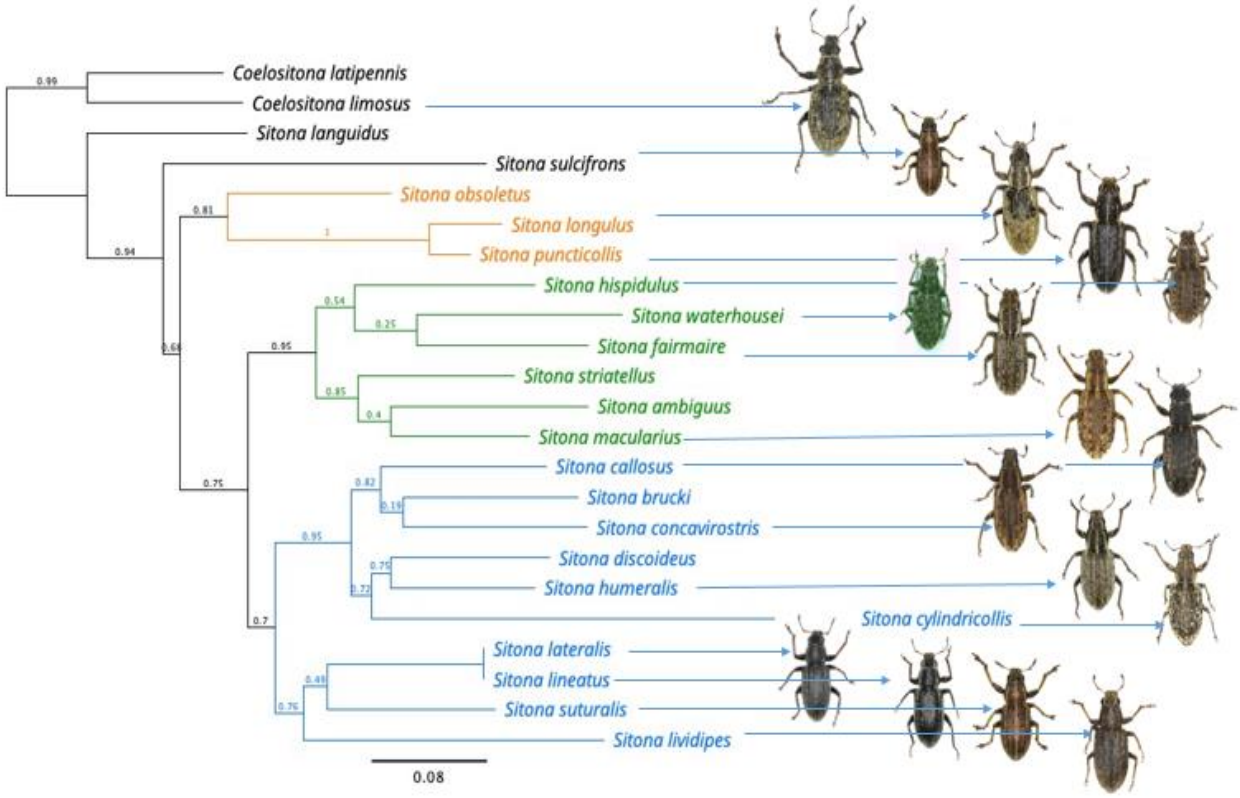

Figure 2. *Maximum Parsimony phylogenetic tree of Sitona species based on cytochrome c oxidase subunit I (COI) sequences. Bootstrap support values (>50%) are shown next to the nodes. Four major clades are highlighted in different colors, with representative adult habitus images provided for each species. Coelositona latipennis and C. limosus were used as outgroup taxa. Scale bar represents genetic distance.*

Sitona languidus. This species was included in Reitter's Setosi group due to the presence of erect setae and a small but present prosternal line. Moreover, this species does not share the ability to feed on

plants of the IRLC group (which includes the majority of temperate herbaceous Leguminosae). This ability is an apomorphic characteristic of *Sitona* (there are only two more exceptions in the genus also from the Setosi group) (Velázquez de Castro et al., 2007). This species was grouped with other Setosi species in Schütten et al. (2023) and in the morphological study by Velázquez de Castro et al., 2007).

Sitona sulcifrons. This species was included in the Laterali group by Reitter (1903), due to the presence of a lateral band of scales and the absence of a precoxal area. Recent studies (Velázquez de Castro et al., **2007**) showed that *S. sulcifrons* has a small precoxal area (p= 55). On the other hand, the sclerites of the internal sac are similar to those of other Laterali species, with large pinnae and forked hamuli, so the morphology is consistent with the grouping within Laterali. However, it also remained in a relatively isolated position in the studies of Stüben et al. (2015) and Schütte et al. (2023).

Group A (Orange color). It includes three species of the Ciliati group. They share a very large precoxal zone (p ranges from 115 to 200), a large body size (5 to 6,5 mm), and the sclerites of the internal sac (pinnae longer than the cucullus, and small hamuli). We propose for this group the name of "*Sitona obsoletus* group", which includes the similar species *Sitona cinnamomeus* Allard, 1863. Stüben et al. (2015) and Schütte et al. (2013) only treated one species of this group, *S. obsoletus*, which was not grouped with other species of *Sitona*. Reitter's Subnudi group probably also belongs to this category, since the precoxal zone is wide (80-83) and the internal sac is similar. It is consistent with the grouping of Velázquez de Castro et al., 2007.

Group B (Green color). This group includes species of *Sitona* with raised setae on the elytra and deep punctures on the pronotum. It includes two of the three *Sitona* species that do not feed in IRLC plants (*S. waterhousei* and *S. striatellus*). Almost all species in group B were included in the Setosi group by Reitter (1903) and grouped together in Velázquez de Castro et al., 2007. But there are two interesting exceptions.

a) *Sitona hispidulus*. Previously included in Angustifrontes due to its narrow frons. However, it has raised setae and deep punctures in the pronotum, two characteristics shared with Setosi. According to the phylogram, these last characters seem more important to cluster *Sitona* species. Therefore, species similar to *S. hispidulus* such as *S. obscuratus* Faust, 1882, belong to this group B.

b) *Sitona striatellus*. Previously included in Convexicolles by Reitter (1903), who thought the precoxal zone was absent. But it was a mistake, as it is present (p= 50). Our phylogram shows the affinity with *Sitona ambiguus* and *S. macularius*. In other studies, these species were also grouped together. In Stüben et al. (2015) *S. macularius* joined *S. ambiguus* and in Schütte et al. (2023) these two species joined *S. striatellus,* as in our phylogram, (but also included *S. languidus,* see comments on the first unclustered species). The internal sac is similar in *S. macularius, S. ambiguus, S. striatellus*, and also the similar species *S. lineellus*. *S. striatellus* also clustered with Setosi species in Velázquez de Castro et al. 2007.

We propose for this group B the name "*Sitona macularius* group of species".

Group C (Blue color). The species in this group do not have raised setae in the elytra, nor deep punctures in the pronotum. This group includes two sister groups, very different morphologically:

Subgroup C1. It includes species from two Reitter groups, Angustifrontes and Callosi, with an evident precoxal zone (75 to 95) and small non-erect setae covering the body. We propose for this group the name *Sitona discoideus* group of species.

Subgroup C2. It includes species of the Ecliliati and Laterali groups (also clustered in Velázquez de Castro et al., 2007). The precoxal zone is very small (25 to 45), there are no dorsal setae, and the

sclerites of the internal sac have very wide hamuli. In addition, the scales of the ventral part of the body are dense and light-coloured. We propose for this group the name *Sitona lineatus* group. The species *Sitona lateralis* and *S. lineatus* showed 100% coincidence in our phylogram. On the other hand, in the study of Stüben et al. (2015) *S. lateralis* showed 100% coincidence with *S. suturalis*. But not with *S. lineatus*. These three species are very close and difficult to separate using molecular methods.

Both subgroups C1 and C2 are similar to those obtained by Stübben et al. (2015) and Schütte et al. (2023), but they did not include *S. concavirrostris* or *S. lividipes* in their studies.

In conclusion, the species group scheme established by Reitter (1903) must be abandoned due to the lack of agreement with our results (table 2). In our phylogenetic approach, *Sitona* were grouped into four groups that can be also morphologically defined. However, more species must be studied to fully clarify the phylogeny of the genus and divide it into subgenera

Table 2. *Groups of Sitona after Reitter (1903)*

Reitter 's group	Characteristic	Precoxal zone	Present status
1. Scutellati	Divergent scutellar scales	variable	*Charagmus*
2. Pubiferi	Absence of dorsal scales	absent	*Coelositona*
3. Oculati	Dorsal keels in rostrum.	absent	*Coelositona*
4. Convexicolles	Deep pronotal punctures	absent	*Sitona* (Group B) + *Andrion*
5. Eciliati	Shallow pronotal punctures	absent	*Sitona* (Group C2)
6. Laterali	Lateral band of scales	absent	*Sitona* (Group C2)
7. Subnudi	Absence of dorsal scales	present	*Sitona* (Not included)
8. Ciliati	Elytral setae absent	present	*Sitona* (Group A)
9. Callosi	Light coloured apical callus of elytra	present	*Sitona* (Group C1)
10. Setosi	Raised dorsal setae	present	*Sitona* (Group B)
11. Angustifrontes	Head with narrow frons	present	*Sitona* (Group C1. Group B)

ACKNOWLEDGEMENTS

This study was financially supported by The Scientific and Technological Research Council of Türkiye with the project number 120O352.

AUTHOR CONTRIBUTIONS

The authors contributed equally to this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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