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Preliminary morphological and molecular study of species of *Saccharicoccus* Ferris, 1950 and *Trionymus* Berg, 1899 (Hemiptera: Coccoidea: Pseudococcidae)

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Abstract. This paper presents preliminary results of morphological and molecular analysis of species of *Saccharicoccus* and *Trionymus*. Five species were analyzed: *Saccharicoccus penium*, *Trionymus aberrans*, *Trionymus isfarensis*, *Trionymus perrisii* and *Trionymus thulensis*. The obtained outcomes show that the occurrence of cryptic species within *T. aberrans* is highly probable. The distinctiveness of other examined species is substantiated. The separateness of *T. isfarensis* and *T. thulensis* is confirmed. Morphological and molecular data suggest that *S. penium* should not be reduced to synonym of *T. isfarensis*.

Key words: entomology, *Saccharicoccus, Trionymus,* morphology, molecular analysis, ITS

INTRODUCTION

The family Pseudococcidae is the second largest family within the superfamily Coccoidea. It consists of over 2200 species (Ben-Dov et al. 2012). The Pseudococcidae is divided into two subfamilies: Pseudococcinae (with 201 genera) and Phenacoccinae (with 69 genera) (Hardy et al. 2008).

One of the largest genera within subfamily Pseudococcinae is *Trionymus* Berg, 1899 and it includes about 120 species (Danzig 1997, Ben-Dov et al. 2012). The genus has worldwide distribution. So far 11 species of *Trionymus* have been recorded in Poland (Koteja & Żak-Ogaza 1989, Łagowska & Koteja 1996, Łagowska 2004, Simon & Herczek 2010). Generally species in this genus live on grasses as well as on herbaceous plants such as *T. multivorus* (Kiritchenko, 1936). They inhabit leaf sheaths

when they live on the grasses, and sometimes roots or leaves. *Trionymus* includes a number of morphologically similar species, which makes their identification difficult because of wide range of variation in the species, and because of this the taxonomic position of some species is unclear (Kosztarab & Kozár 1988).

The morphology and biology of adult females of *Trionymus* are similar to the females of Palearctic *Balanococcus* Williams, 1962. Species of *Balanococcus* differ in having tubular ducts with a deep collar covering the duct, short antennae, poorly developed ostioles and comparatively large hind coxae (Gavrilov 2010). Some species of *Trionymus* are also close to species of *Dysmicoccus* Ferris, 1950, which differ in possessing more than 5 or 7 pairs of cerarii (Kosztarab & Kozár 1988).

The whole group comprising species of *Balanococcus*, *Dysmicoccus* and *Trionymus* needs a thorough revision (Kosztarab & Kozár 1988; Gavrilov & Trapeznikova 2007).

Taxonomical problem concerns also systematic position of species within genus *Saccharicoccus* Ferris, 1950. It consists of only two species: *S. penium* Williams, 1962 and *S. sacchari* (Cockerell, 1895) (Kosztarab & Kozár 1988, Tang 1992). Only the former, which has been collected from grasses, occurs in Poland. *Saccharicoccus penium* Williams, 1962 was synonymized with *Trionymus isfarensis* (Borchsenius, 1949) by Danzig (1983). Koteja (1986) did not accept this procedure and decision. Systematic position of this species is still under discussion.

Recently, molecular studies focused on the use of DNA markers that can be used to distinguish between closely related taxa. Among molecular markers used for species identification of mealybugs the following genetic markers are applied: ITS, COI and 28S (Downie & Gullan 2004, Gullan et al. 2010, Malausa et al. 2011). The present paper shows the preliminary results of molecular investigations conducted with the application of ITS2.

These investigations attempt to establish taxonomical position of selected species of *Saccharicoccus* and *Trionymus* and to detect cryptic species within these genera using both morphological and molecular data.

MATERIAL AND METHODS

Scale insects were collected by using hand collecting method (Table 1). Mealybugs samples were preserved in 96-100% ethanol until DNA extraction. Material was collected and identified by Kalandyk-Kołodziejczyk M. and Simon E. Representatives of five species were used in morphological and molecular analysis: *Saccharicoccus penium* Williams, 1962, *Trionymus aberrans* Goux, 1938, *Trionymus isfarensis* (Borchsenius, 1949), *Trionymus perrisii* (Signoret, 1875), *Trionymus thulensis* Green, 1931. Characteristic morphological features of examined species and number of analyzed specimens are presented in Table 2.

The sequences of *Dysmicoccus brevipes* (Cockerell, 1893) (GenBank Accesion: Gu134673) were used as an outgroup due to morphological similarities among species of *Dysmicoccus* and *Trionymus*.

Digital images were obtained with a DN-100 camera installed in a Nikon Eclipse-E600 light microscope.

Total genomic DNA was isolated using DNeasy Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. A fragment of the ITS2 region was studied. The primers used for PCR amplifications were ITS2-M-F and ITS2-M-F (Malausa et al. 2011). ITS2 was amplified as follows: initial denaturation at 98°C for 30s, followed by 35 cycles of 98°C for 10s; annealing temperatures of 58°C for 15s; extension at 72°C for 15s; final extension at 72°C for 5 min. PCR products were purified using a QIAquick® PCR purification Kit (QIAGEN) and sequenced directly using an automated sequencer (Genome Sequencer GS FLX Roche). The studied sequences were deposited in GenBank under accession numbers given in Table 1., where it is presented together with the accession numbers of the sequences used for the phylogenetic analysis.

Chromatograms, resulting from sequencing of both strands, were analysed using Chromas V2.3 (Technelysium Pty Ltd., 2004) software. Alignments for the studied sequence were made using Clustal X (Thompson et al. 1997).

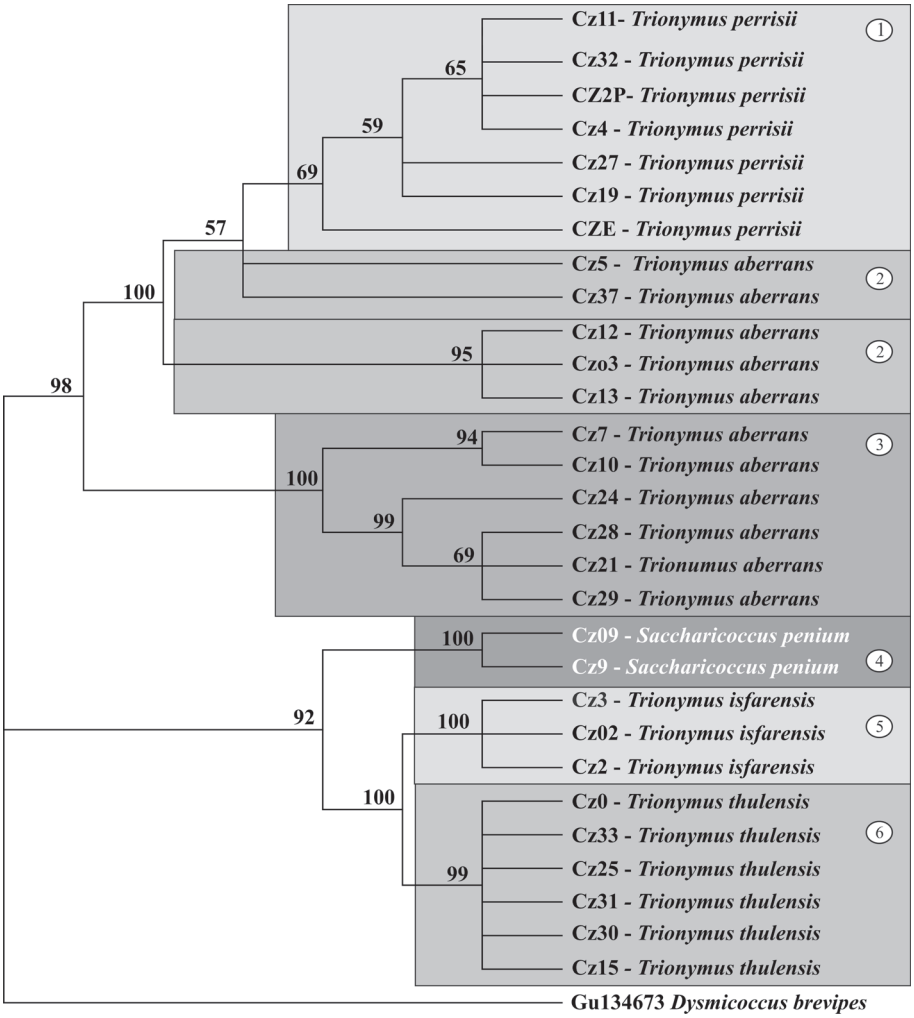
Tree reconstructions based on nucleotide sequences were carried out by maximum-likelihood (ML) method as implemented in MEGA version 5 (Tamura et al. 2011).

RESULTS

The tree reconstructions based on nucleotide sequences the Internal Transcriber Spacer region of the nuclear ribosomal DNA were carried out by the maximum-likelihood (ML) method (Fig.1). The ML tree was obtained using the Tamura 3-parameter model (T92) (Tamura 1992), because it scored the lowest BIC (Bayesian Information Criterion) value when different models of substitution pattern were tested. Branches corresponding to partitions reproduced in less than 55% bootstrap replicates were collapsed.

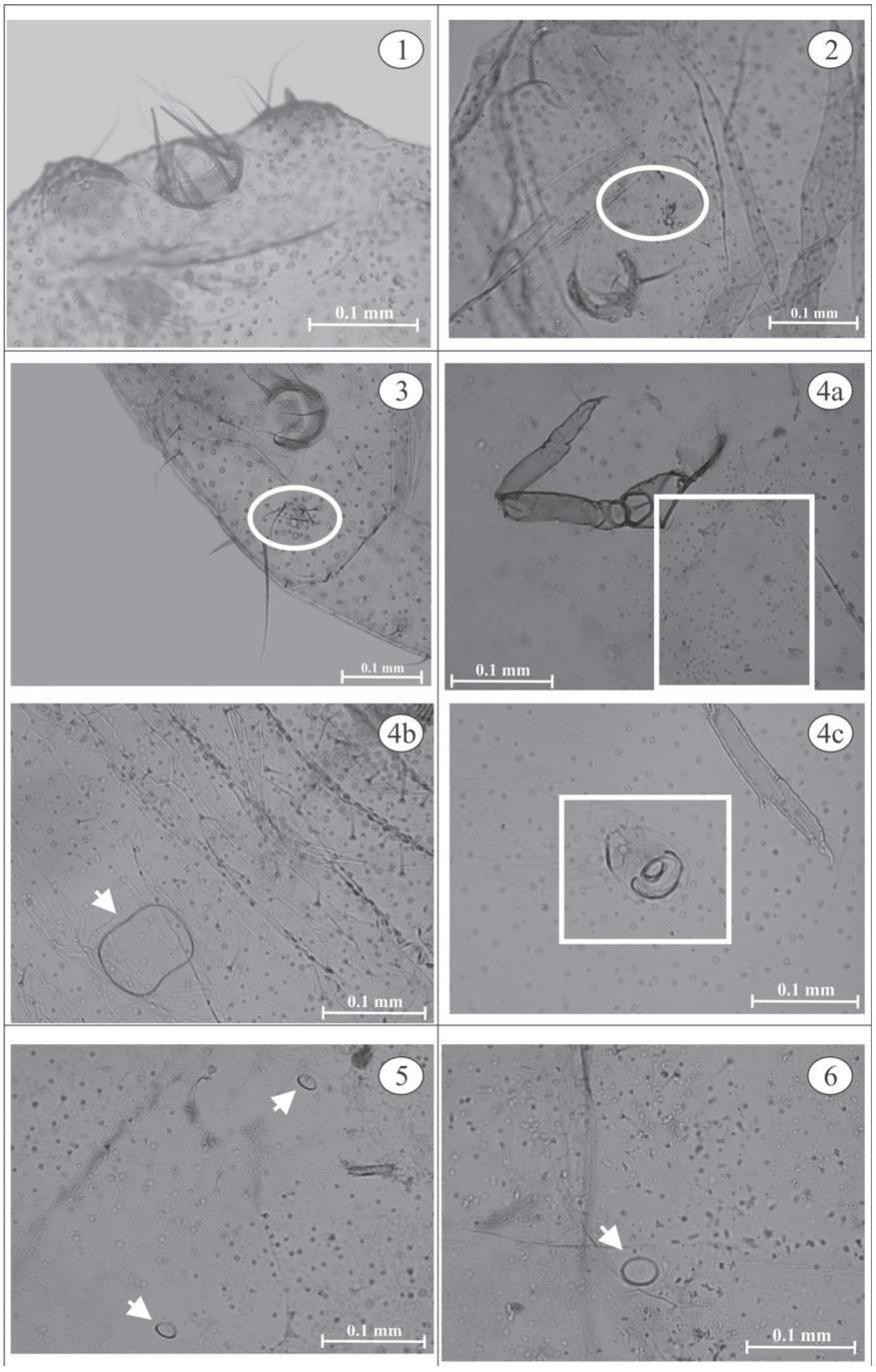
Morphological variability within examined specimens of *Trionymus perrisii* was not observed. All analysed specimens belonging to this species possess last pair of cerarii with 38-40 trilocular pores situated on large sclerotized plate (Fig. 2.1.)

Analysed tree comprises two main clades. The first consists of two species: *Trionymus perrisii* and *Trionymus aberrans*. Some individuals of *T. aberrans* constitute a separate clade (no.3 in Fig.1) supported by maximum bootstrap value and some form a clade with *T. perrisii* (no.1 in Fig.1). Specimens of *T. aberrans* marked with no.2 (Cz5, Cz37,Cz12,Czo3, Cz13) possess some features distinguishable from specimens that form clade no.3: hind coxae with very few translucent pores and a smaller number of trilocular pores in the last pair of cerarii (Fig.2.2). These individuals were collected in the area of postindustrial wastelands. Specimens constituting clade no.3 possess numerous translucent pores in hind coxae and higher number of trilocular pores in the last pair of cerarii (Fig. 2.3). Specimens (Cz21, Cz28, Cz29) collected in Austria are distinguishable by the presence of higher number (15 - 16) of trilocular pores in the anal lobe cerarii. The others possess from 8 to 12 pores. All specimens identified as *T. aberrans* possess characteristic horseshoe-shaped anal ring (Fig. 2.2., Fig. 2.3.), 8-segmented antennae, two pairs of cerarii and numerous trilocular pores on both body surfaces.



1. Results of Maximum Likelihood of ITS sequences. Bootstrap values are indicated on the branches when higher than 55

2. Characteristic features of examined species: 2.1.Cerarii with large number of trilocular pores in *Trionymus perrisii*; 2.2.Cerarii with few trilocular pores in the specimen of *Trionymus aberrans* forming a clade with *T. perrisii*; 2.3.Cerarii with many trilocular pores in the specimen of *T. aberrans* which forms clade comprising only *T. aberrans*; 2.4.Characteristic features of *Saccharicoccus penium*: 2.4.a. minute pores around hind leg, 2.4.b. hourglass-like circulus, 2.4.c. spiracle; 2.5.Two circuli in *Trionymus isfarensis*; 2.6.The only round circulus in *Trionymus thulensis* (see next page)


1
0.1 mm
2
0.1 mm
3
0.1 mm
4a
0.1 mm
4b
0.1 mm
4c
0.1 mm
5
0.1 mm
6
0.1 mm

Second main clade comprises three species forming two sister clades. First of them consists of *Saccharicoccus penium* (no.4). All specimens identified as *S. penium* are characterized by some distinctive morphological features, including well visible numerous simple minute pores around the hind legs (Fig. 2.4a), elongate hourglass-like circulus located between abdominal segments 4 and 5 (Fig. 2.4b) and spiracles of characteristic shape (Fig. 2.4c).

Second sister clade comprises two species*: Trionymus isfarensis* (no.5) and *Trionymus thulensis* (no.6). Separateness of these species is supported by high bootstrap value. They differ from each other with some distinctive features: number of circuli (2-3 in *T. isfarensis*; 1 in *T. thulensis* - Fig.2.5-2.6.); number of hairlike setae in the last pair of cerarii (3-5 in *T. isfarensis;* 1 in *T. thulensis*).

DISCUSSION

Our study shows that *Trionymus aberrans* seems to contain cryptic species complex. *T. aberrans* was described and illustrated by Goux (1938) and analysed in details by many other authors (e.g. Borchsenius 1949, Tereznikova 1975, Danzig 1997). It was characterized by 8-segmented antennae, horseshoe-shaped anal ring and absence of circulus. Morphological variability within this species connected with number of antennal segments was observed by Goux (1938). Two subspecies: *T. aberrans aberrans* and *T. aberrans ovalis* were described by this author. Members of the latter were characterized by possessing 9-segmented antennae (Goux 1941). This feature was not observed in any of the examined specimens. Tang (1992) regarded this subspecies a synonym of *T. aberrans*.

It is supposed that differences between specimens occurring in the area of postindustrial wastelands and others collected in differential habitats (meadows, xerothermic grasslands and forests) might be caused by environmental conditions. The effect of environmental conditions on variation of characters in different species was noted by Williams (1985). Apart from Goux (1938, 1941) morphological variability in *T. aberrans* was observed by Kosztarab & Kozár, 1988 (presence or absence of circulus), which indicates that occurrence of cryptic species classified till now as *T. aberrans* is highly probable.

Morphological studies indicate that *S. penium* should not be reduced to synonym of *T. isfarensis* as Danzig (1983) suggested. Koteja (1986) did not approve her concept. He emphasized that *T. isfarensis* as described by Borchsenius (1949) is entirely different from *S. penium* taking into consideration the number and shape of circuli, occurrence of minute pores around hind legs and the structure of spiracles. All examined individuals classified as *S. penium* are characterized by presence of typical features described by Williams (1962), which are not visible in specimens classified as *T. isfarensis*. Our morphological analysis shows that *S. penium* and *T. isfarensis* are separate species. Koteja (1986) suggested that specimens collected previously in Poland (Koteja & Żak-Ogaza 1969, 1983, Koteja 1971) and included to *T. isfarensis* had been misidentified. In his later papers one can find the information on the new localities of *T. isfarensis* in

Poland (Koteja & Żak-Ogaza 1989; Łagowska & Koteja 1996). Recently *T. isfarensis* was noted in the eastern part of the Tarnowskie Góry Hummock, which is a region located in the southern Poland (Kalandyk & Węgierek 2010).

Genus *Saccharicoccus* was considered to be close to *Trionymus*. According to Kosztarab and Kozár (1988) species of *Trionymus* possess following features: antennae 6-8 segmented, 4 dorsal ostioles (rarely 2) present, hind coxae often with translucent pores, fewer than 6 pairs of cerarii, trilocular pores on both body surfaces, multilocular pores always on venter, often present on dorsum, oral tubular ducts on both body surfaces. Typical features of *S. penium* are: 7-segmented antennae, ostioles poorly developed, hind coxae with a few translucent pores, one pair of cerarii, trilocular pores evenly distributed on both body surfaces, multilocular pores occurring on venter and dorsum of abdomen, numerous trilocular pores on both body surfaces, 2 sizes of tubular ducts (Williams 1962). Result of our preliminary molecular studies does not explain if *S. penium* should be transferred to genus *Trionymus*. It only shows that *S. penium* and *T. isfarensis* should not be synonymized.

Other genera similar to *Trionymus* are *Balanococcus* and *Dysmicoccus* (Danzig, 1997, Kosztarab & Kozár 1988). Some species of *Trionymus* are characterized by short tubular duct arranged together with multilocular pores which brings them very close to *Balanococcus* Williams, 1962. The *Dysmicoccus-Trionymus* complex includes several (perhaps 3) groups of species (Kosztarab & Kozár 1988).

Although *T. perrisii* is a species considered to be morphologically variable (Kosztarab & Kozár 1988), we did not notice the variability within this species.

CONCLUSION

On the basis of molecular analysis based on ITS the existence of cryptic species within *T. aberrans* is highly probable. These species form a clade with *T. perrisii.* Morphological variability within *T. aberrans* may be connected with the environmental conditions, a statement that should be tested using higher number of individuals.

Molecular investigations confirm separateness of other examined species: *S. penium, T. isfarensis* and *T. thulensis*, which is in accordance with an analysis of the morphological features. The obtained preliminary results should be tested with the application of other molecular markers.

Since the applied molecular marker ITS is not useful in systematic and phylogeny but is the most informative for study of cryptic taxa and delimitation of species (Malausa et.al. 2011), we cannot conclude about taxonomic affinity of *S. penium.* The results of molecular and morphological research suggest that *S.penium* is not a synonym of *T. isfarensis,* but its affinity to genus *Saccharicoccus* or *Trionymus* is still debated. Its unclear systematic position will be determined during further research. It is necessary to test the species of this two taxa: *S. penium* and *T. isfarensis* by means of using a large number of genetic indicators.

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Table 1. The collection data of the studied material

Species	Voucher number	Collection site		Host plant	GenBank Accession No.
Trionymus perrisii	Cz11	Rudy Wielkie, Poland 50° 12' 09" N; 18° 26' 37" E	26.09.11	*Festuca ovina*	KC631169
T. perrisii	Cz32	Rudy Wielkie, Poland 50° 12' 09" N; 18° 26' 37" E	26.09.11	*Festuca ovina*	KC631170
T. perrisii	Cz2P	Rudy Wielkie, Poland 50° 12' 09." N; 18° 26' 37" E	02.08.11	*Festuca ovina*	KC631171
T. perrisii	Cz4	Katowice, Poland 50° 15' 13" N; 19° 02' 47" E	15.09.11	*Phleum pratense*	KC631172
T. perrisii	Cz27	Nowa Wieś, Poland 50° 27' 10" N; 19° 05' 31" E	05.10.11	*Phleum pratense*	KC631168
T. perrisii	Cz19	Neudorlf, Austria 47° 47' 17" N; 16° 17' 17" E	02.10.11	*Calamagrostis epigejos*	KC631173
T. perrisii	CzE	Preganziol, Italy 45° 35' 29"N; 12° 13' 58"E	03.10.11	*Festuca* sp.	KC631174
Trionymus aberrans	Cz5	Ruda Śląska, Poland 50° 16' 02" N; 18° 52' 09" E	25.09.11	*Agrostis capillaris*	KC631166
T. aberrans	Cz37	Ruda Śląska, Poland 50° 16' 02" N; 18° 52' 09" E	25.09. 11	*Agrostis capillaris*	KC631167
T. aberrans	Cz12	Piekary Śląskie, Poland 50° 22' 00" N; 18° 58' 17"E	29.09.11	*Deschampsia caespitosa*	KC631163
T. aberrans	Cz03	Piekary Śląskie, Poland 50° 22' 00" N; 18° 58' 17"E	29.09.11	*Deschampsia caespitosa*	KC631164

Table 1. The collection data of the studied material (cont.)

T. aberrans	Cz13	Piekary Śląskie, Poland 50° 22' 00" N; 18° 58' 17" E	29.09.11	*Deschampsia caespitosa*	KC631165
T. aberrans	Cz7	Katowice, Poland 50° 15' 13" N; 19° 02' 47" E	12.08.11	*Phleum pratense*	KC631175
T. aberrans	Cz10	Katowice, Poland 50° 15' 13" N; 19° 02' 47" E	16.09.11	*Agrostis capillaris*	KC631176
T. aberrans	Cz24	Twardowice, Poland 50° 25' 03" N; 19° 04' 36" E	01.10.11	*Agrostis capillaris*	KC631179
T. aberrans	Cz28	Neudorlf, Austria 47° 47' 17" N; 16° 17' 17" E	02.10.11	*Festuca* sp.	KC631178
T. aberrans	Cz21	Neudorlf, Austria 47° 47' 17" N; 16° 17' 17" E	02.10.11	*Festuca* sp.	KC631177
T. aberrans	Cz29	Neudorlf, Austria 47° 47' 17" N; 16° 17' 17" E	02.10.11	*Festuca* sp.	KC631180
Saccharicoccus penium	Cz09	Dąbrowa Górnicza, Poland 50° 23' 15" N; 19° 17' 33" E	20.07.11	*Poa compressa*	KC631181
S. penium	Cz9	Dąbrowa Górnicza, Poland 50° 23' 15" N; 19° 17' 33" E	20.07.11	*Poa compressa*	KC631182
Trionymus isfarensis	Cz3	Piekary Śląskie, Poland 50° 21' 11" N; 19° 00' 10" E	30.09.11	*Agrostis capillaris*	KC631161

Table 1. The collection data of the studied material (cont.)

T. isfarensis	Cz02	Piekary Śląskie, Poland 50° 21' 11" N; 19° 00' 10" E	10.07.11	*Agrostis capillaris*	KC631160
T. isfarensis	Cz2	Piekary Śląskie, Poland 50° 21' 11" N; 19° 00' 10" E	23.07.11	*Agrostis capillaris*	KC631162
Trionymus thulensis	Cz0	Ujudvar, Hungary 47° 50' 10" N; 17° 14' 00" E	28.09.11	*Arrhenatherum elatius*	KC631155
T. thulensis	Cz33	Ujudvar, Hungary 47° 50' 10" N; 17° 14' 00" E	28.09.11	*Arrhenatherum elatius*	KC631158
T. thulensis	Cz25	Ujudvar, Hungary 47° 50' 10" N; 17° 14' 00" E	28.09.11	*Arrhenatherum elatius*	KC631154
T. thulensis	Cz31	Ujudvar, Hungary 47° 50' 10" N; 17° 14' 00" E	28.09.11	*Arrhenatherum elatius*	KC631157
T. thulensis	Cz30	Ujudvar, Hungary 47° 50' 10" N; 17° 14' 00" E	28.09.11	*Arrhenatherum elatius*	KC631156
T. thulensis	Cz15	Ujudvar, Hungary 47° 50' 10" N; 17° 14' 00" E	28.09.11	*Arrhenatherum elatius*	KC631159

Table.2. Morphological features of the examined species of *Saccharicoccus* and *Trionymus*

species	*Trionymus perrisii*	*Trionymus aberrans* forming clade with *Trionymus perrisii* (Cz5, Cz37, Cz12, Czo3, Cz13)	*Trionymus aberrans* typical	*Trionymus aberrans* forming clade 3	*Saccharicoccus penium*	*Trionymus isfarensis*	*Trionymus thulensis*
number of analyzed specimens	18	15		16	8	6	10
number of antennae segments	8	8	8	8	7	7	7 - 8
appearence of legs	well developed	slender	slender	slender	slender	well developed	slender
number of translucent pores in hind coxae	numerous	few	numerous	numerous	few	few	few
number of pairs of cerarii	2	2	2	2	1	from 1 to 2	2
presence of sclerotized plates in C18	present, large	-	-	-	-	present, small	-
number of trilocular pores in C18	38-40	6 - 8	8 - 12	8 - 16	7 - 9	6 - 7	7 - 9
number of hairlike setae in C18	9 - 13	3 - 4	3 - 4	3 - 4	1	3 - 5	1
presence of circuli	present	absent	absent	absent	present	present	present
number of circuli	1	0	0	0	1	2 - 3	1

Table.2. Morphological features of the examined species of *Saccharicoccus* and *Trionymus* (cont.)

species	*Trionymus perrisii*	*Trionymus aberrans* forming clade with *Trionymus perrisii* (Cz5, Cz37, Cz12, Czo3, Cz13)	*Trionymus aberrans* typical	*Trionymus aberrans* forming clade 3	*Saccharicoccus penium*	*Trionymus isfarensis*	*Trionymus thulensis*
shape of circuli	oval	–	–	–	oval, hourglass-shape	one between segments 3 and 4 large with sides notched, others small	round and small
shape of anal ring	subcircular	horseshoe-shaped	horseshoe-shaped	horseshoe-shaped	oval	almost circular	almost circular
distinguishable feature	C18 on large sclerotized plates	horseshoe-shaped anal ring	horseshoe-shaped anal ring	horseshoe-shaped anal ring	minute simple pores around hind legs, hourglass shape circulus	a semi-circle iof trilocular pores in front of each spiracle, oral collar ducts associated with multilocular pores	each spiracle surrounded by a few trilocular pores