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An Unusual Symbiotic System in *Elymana kozhevnikovi* (Zachvatkin, 1938) and *Elymana sulphurella* (Zetterstedt, 1828) (Insecta, Hemiptera, Cicadellidae: Deltocephalinae)

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Original article

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Morphological and molecular analyses revealed that the Deltocephalinae leafhoppers *Elymana kozhevnikovi* and *E. sulphurella* are host to four bacteriocyte-associated microorganisms: *Sulcia* (phylum Bacteroidetes), *Nasuia* (phylum Proteobacteria, class Betaproteobacteria), *Arsenophonus* (phylum Proteobacteria, class Gammaproteobacteria) and *Sodalis*-like bacteria (phylum Proteobacteria, class Gammaproteobacteria). Ultrastructural observations showed that in some bacteriocytes, apart from *Sulcia,* small elongated, rod-shaped bacteria are likewise present. The use of fluorescence *in situ* hybridization (FISH) revealed the occurrence of *Sodalis*-like bacteria in these bacteriocytes. *Sodalis*-like bacteria were also distributed in some cells of the bacteriome sheath. *Nasuia* and *Arsenophonus* co-existed in the same bacteriocytes. Moreover, *Arsenophonus* bacteria were dispersed in fat body cells. *Wolbachia* and *Rickettsia* were also detected alongside bacteriocyte-associated symbionts in *E. kozhevnikovi* and *E. sulphurella*. *Sulcia*, *Nasuia*, *Arsenophonus* and *Sodalis*-like bacteria are transovarially transmitted from one generation to the next.

Key words: Symbiotic microorganisms, *Sulcia*, *Nasuia*, *Arsenophonus*, *Sodalis*-like bacteria, *Wolbachia*, *Rickettsia*, transovarial transmission.

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Deltocephalinae leafhoppers, as other plant sap-sucking hemipterans, live in mutualistic relations with microorganisms including bacteria and/or yeast-like symbionts. The presence of these associates is connected with the restricted diet of host insects, poor in essential nutrients (mainly amino acids) (reviewed e.g. in BUCHNER 1965; DOUGLAS 1998; BAUMANN 2005). It is generally accepted that the occurrence of obligate symbionts is a result of an ancient acquisition of microorganisms by the ancestor of these insects resulting in the presence of microorganisms in all the descendants. As a consequence of the long-term co-evolution between host insects and their symbionts, neither can survive as separate entities (i.e. host insects devoid of microorganisms cannot properly develop or reproduce, microorganisms cannot be cultivated on laboratory media). On account of this mutualistic relationship, BUCHNER (1965) termed the obligate microorganisms "primary symbionts". BUCHNER (1965) also distinguished "accessory symbionts" (later termed "facultative symbionts" or "secondary symbionts") which may occur in some populations only. The presence of the latter in host insects is a consequence of, as a rule, their more recent acquisition. Secondary symbionts may fulfill different functions, e.g. they may protect host insects against parasites or heat stress (MONTLLOR *et al.* 2002; OLIVER *et al*. 2003; ŁUKASIK *et al*. 2013). Recent genomic analyses have shown that they may also be engaged in the synthesis of amino acids or other factors in metabolic pathways in different groups of hemipterans (TAKIYA *et al.* 2006; LAMELAS *et al.* 2011; SLOAN & MORAN 2012; LUAN *et al*. 2015; HUSNIK & MCCUTCHEON 2016). On ac-

count on the metabolic complementarity of symbionts residing in auchenorrhynchous hemipterans and their mutualistic association with host insects, TAKIYA and co-workers (2006) termed these symbionts "coprimary symbionts".

Previous histological observations by MÜLLER (1962) and BUCHNER (1965) have shown that auchenorrhynchous hemipterans (cicadas, leafhoppers, treehoppers, spittlebugs and planthoppers) generally harbor several different symbionts. More recent molecular work determined their systematic affinity and function, revealing that auchenorrhynchous hemipterans host the ancient symbionts: Candidatus *Sulcia muelleri* (hereafter *Sulcia*) (phylum Bacteroidetes) and betaproteobacterial symbionts (phylum Proteobacteria) (MORAN *et al.* 2005; TAKIYA *et al*. 2006; BRESSAN *et al*. 2009; NODA *et al*. 2012; URBAN & CRYAN 2012; BENNETT & MORAN 2013; ISHII *et al.* 2013; KOGA *et al.* 2013; SZKLARZEWICZ *et al.* 2016; KOBIAŁKA *et al.* 2015, 2016; MAO *et al*. 2017). In some auchenorrhynchous hemipterans, the ancient betaproteobacterial symbiont has been lost and replaced by other bacteria (e.g. Gammaproteobacteria or Alphaproteobacteria) (MORAN *et al.* 2003; TAKIYA *et al.* 2006; MCCUTCHEON *et al.* 2009; KOGA *et al*. 2013; MICHALIK *et al*. 2014). Moreover, in some groups of auchenorrhynchous hemipterans, the ancient symbionts (only the betaproteobacterial symbiont or both *Sulcia* and the betaproteobacterial symbiont) have been eliminated and replaced by yeast-like microorganisms (NODA 1977; NODA *et al.* 1995; SACCHI *et al.* 2008; MICHALIK *et al.* 2009; NISHINO *et al.* 2016; KOBIAŁKA *et al.* 2016, 2017). Since the Deltocephalinae possess diverse symbiotic associates in comparison to other auchenorrhynchous hemipterans (SACCHI *et al.* 2008; ISHII *et al.* 2013; BENNETT & MORAN 2013; KOBIAŁKA *et al.* 2015, 2016, 2017), in this work we described the symbiotic systems of two unexamined representatives of these leafhoppers, *Elymana kozhevnikovi* and *E. sulphurella,* by means of molecular and ultrastructural methods.

Material and Methods

Insects

The adult females of *Elymana kozhevnikovi* (Zachvatkin, 1938) and *Elymana sulphurella* (Zetterstedt, 1828) were collected in the southern part of Poland (Kraków, Częstochowa, Gliwice, Katowice) from June to September 2012-2016 from the grasses of the family Poaceae.

Light and electron microscopy

The dissected abdomens of adult females of all species examined were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for three months. Next, the material was rinsed in the phosphate buffer with the addition of sucrose (5.8 g/100 ml) and postfixed in 1% osmium tetroxide in the same buffer. Then, the material was dehydrated in a graded series of ethanol and acetone and embedded in epoxy resin Epon 812 (Serva, Heidelberg, Germany). The semithin sections (1 µm thick) obtained from about twenty five females of *E. kozhevnikovi* and about twenty five females of *E. sulphurella* were stained with 1% methylene blue in 1% borax and photographed using a Nikon Eclipse 80i light microscope (LM). The ultrathin sections (90 nm thick) were contrasted with uranyl acetate and lead citrate and examined using a Jeol JEM 2100 electron transmission microscope (TEM) at 80 kV.

DNA analyses

DNA was extracted individually from the dissected abdomens of ten females preserved in 100% ethanol. DNA extraction was conducted using the Sherlock AX extraction kit (A&A Biotechnology) according to the manufacturer's protocol and next DNA was stored at 4°C for further analyses. Molecular identification of bacteria associated with examined species was performed based on their 16S rDNA sequences which were obtained by amplifications with symbiont-specific primers (listed in Table 1). PCR reactions were run in a total volume of 20 µl made up of 10 µl of the PCR Mix Plus HGC mixture (A&A Biotechnology), 8 µl of water, 0.5 µl of each of the primers (10 µM) and 1 µl of the DNA template (1 µg/µl) under the following protocol: an initial denaturation step at 94°C for a duration of 3 min, followed by 33 cycles at 94°C for 30 s, 54-56°C for 40 s (see Table 1), 70°C for 1 min 40 s and a final extension step of 5 min at 72°C. The PCR products were visualized by electrophoresis in 1.5% agarose gel stained with Midori Green (Nippon Genetics Europe). The positive PCR products were sent to an external company (Genomed) for DNA sequencing. The GenBank accession numbers of sequences obtained are listed in Table 2.

Phylogenetic analyses

The phylogenetic analyses were performed based on sequences of 16S rDNA of symbionts of *E. kozhevnikovi* and *E. sulphurella* and selected symbionts of Deltocephalinae leafhoppers deposited in the GenBank database. The sequences were then edited using BioEdit Sequence Alignment

Table 1

Primers and fluorochrome-labeled probes used in this study

Purpose	Primer name	Primer sequence (5'-3')	Target gene	Annealing temperature	Source
Diagnostic PCR	10CFB	AGAGTTTGATCATGGCTCAGGATG	16S rRNA gene of *Sulcia*	54°C	MORAN *et al.* 2005
	CFB1515R	GTACGGCTACCTTGTTACGACTTAG			
	16SA1	AGAGTTGATCMTGGCTCAG	16S rRNA gene of *Sodalis*-like symbionts	54°C	FUKATSU & NIKOH 1998
	Sod1248R	TCCGCTGACTCTCGCGAGAT			
	ArsF	TGGCTCAGATTGAACGCTG	16S rRNA gene of *Arsenophonus*	54°C	This study
	ArsR	CACCGCAGTCATGAATCAC			
	Nasuia2F	TAAAGCGGGGAAAACCTCGT	16S rRNA gene of *Nasuia*	56°C	KOBIAŁKA *et al.* in preparation
	Nasuia2R	GCATGCTGATCCGCGATTAC			
	Nasuia5F	GCTTGATCCAGCAATGTYRC	16S rRNA gene of *Nasuia*	56°C	KOBIAŁKA *et al.* in preparation
	Nasuia5R	ACCTTCCAGTACGGCTACCT			
	Rick158F	CGGAGG AAAGAT TTATCG CTG	16S rRNA gene of *Rickettsia*	54°C	ISHII *et al*. 2013
	Rick1206R	CACGTC ACCGTC TTGCTC			
	WspF	TGGTCCAATAAGTGAGAGAAAC	16S rRNA gene of *Wolbachia*	55°C	ZHOU *et al*. 1998
	WspR	AAAAATTAAACGCTACTCCA			
FISH	Ars2	Cy5-TCATGACCACAACCTCCAAA	16S rRNA gene of *Arsenophonus*	Not applicable	GOTTLIEB *et al*. 2008
	BET940R	Cy5-TTAATCCACATCATCCACCG	16S rRNA gene of Betaproteobacteria	Not applicable	DEMANÈCHE *et al.* 2008
	Sod1248R	Cy3-TCCGCTGACTCTCGGGAGAT	16S rRNA gene of *Sodalis*-like symbionts	Not applicable	KOGA *et al*. 2013
	Sul664R	Cy3-CCMCACATTCCAGYTACTCC	16S rRNA gene of *Sulcia*	Not applicable	KOGA *et al*. 2013

Table 2

List of investigated symbiotic microorganisms with the accession numbers of the sequences

Species	Symbionts	GenBank number
Elymana kozhevnikovi (Zachvatkin, 1938)	*Sulcia*	MG840399
	Nasuia	MG878963
	Arsenophonus	MG894669
	Sodalis	MG835278
	Wolbachia	MG873552
Elymana sulphurella (Zetterstedt, 1828)	*Sulcia*	MG840400
	Nasuia	MG878964
	Arsenophonus	MG894670
	Sodalis	MG835279
	Wolbachia	MG873553

Editor 5.0.9 (HALL 1999), and following this, the sequence alignments were generated using ClustalX 1.8 (THOMPSON *et al*. 1997). The phylogenetic analyses were conducted using MrBayes 3.2.2 software (HUELSENBECK & RONQUIST 2001). In this analysis four incremental Metropolis-coupled MCMC chains (3 heated and 1 cold) were run for ten million generations with sampling every 1000 generations. The convergence of analyses was validated using Tracer software (RAMBAUT & DRUMMOND 2007) and the first 25 % of trees were discarded as 'burn-in'. The results of the Bayesian analysis were visualized using FigTree 1.4.0 software (RAMBAUT 2009).

Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) was conducted with symbiont-specific probes (see Table 1). Ten females preserved in 100% ethanol were rehydrated, fixed in 4% formaldehyde and dehydrated through incubations in 80%, 90% and

100% ethanol and acetone. Then, material was embedded in Technovit 8100 resin and cut into sections. Hybridization was performed using a hybridization buffer containing: 1 ml 1M Tris-HCl (pH 8.0), 9 ml 5 M NaCl, 25 µl 20% SDS, 15 ml 30% formamide and about 15 ml of distilled water. The slides were incubated in 200 µl of hybridization solution (hybridization buffer + probes) overnight, at room temperature (ŁUKASIK *et al.* 2017). Next, the slides were washed in PBS three times for 10 minutes, dried and covered with ProLong Gold Antifade Reagent (Life Technologies). The hybridized slides were then examined using a confocal laser scanning microscope Zeiss Axio Observer LSM 710.

Results

Molecular identification of symbiotic microorganisms

Analysis of the 16S rDNA sequences of symbionts associated with *Elymana kozhevnikovi* and *Elymana sulphurella* indicated that the examined species of deltocephalinae leafhoppers are host to six kinds of bacteria: *Sulcia*, *Nasuia*, *Arsenophonus*, *Sodalis*, *Wolbachia* and *Rickettsia*. *Sulcia*, *Nasuia*, *Arsenophonus* and *Sodalis* were detected in all the examined individuals. The 16S rDNA sequences of *Sulcia*, *Nasuia* and *Arsenophonus* symbionts of both *Elymana* species were identical. Sequences of *Sulcia* and *Nasuia* show a high similarity (99%) to 16S rDNA sequences of *Sulcia* and *Nasuia* occurring in other representatives of Deltocephalinae, whereas 16S rDNA sequences of *Arsenophonus* symbionts are similar to those in *Arsenophonus* bacteria detected in the bat fly *Basilia boardmani* [KC597734] and aphid *Aphis melosae* [KF824532]. In turn, the sequences of 16S rDNA of *Sodalis*-like bacteria of *E. kozhevnikovi* and *E. sulphurella* are almost identical (99% identity) and display 99% similarity to the 16S rDNA of bacteria *Sodalis praecaptivus* [CP006569] and the *Sodalis* symbiont of the clown stink bug *Poecilocoris lewisi* [AB915782]. In some of the examined individuals, bacteria belonging to the genera *Wolbachia* (*E. kozhevnikovi* 2/6; *E. sulphurella* 2/8) and *Rickettsia* (*E. kozhevnikovi* 3/7; *E. sulphurella* 3/7) were also detected.

Phylogenetic analyses of the obtained 16S rDNA sequences of *Sulcia* and *Nasuia* symbionts confirmed their systematic affiliation (Figs 1, 2). The topologies resulting from the Bayesian inference of the *Sulcia* and *Nasuia* symbionts are shown in Figs 1 and 2, respectively.

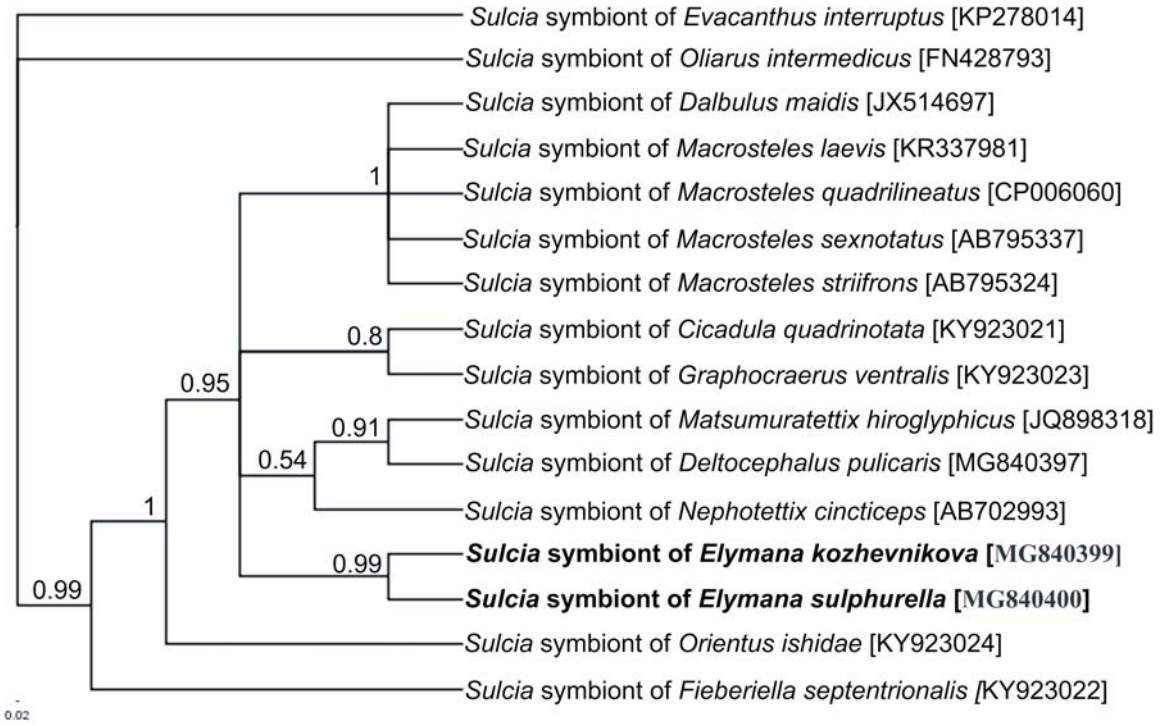


Fig. 1. Phylogenetic tree showing the relationships of *Sulcia* symbionts of the examined *Elymana kozhevnikovi* and *E. sulphurella* leafhoppers and other representatives of the subfamily Deltocephalinae, based on 16S RNA gene sequences. The numbers associated with the branches indicate the Bayesian posterior probability values. The accession numbers of the sequences used in the phylogenetic analysis have been put in brackets. For outgroups, *Sulcia* symbionts of the planthopper *Oliarus intermedicus* (Fulgoromorpha) and leafhopper *Evacanthus interruptus* (Cicadellidae) were used.

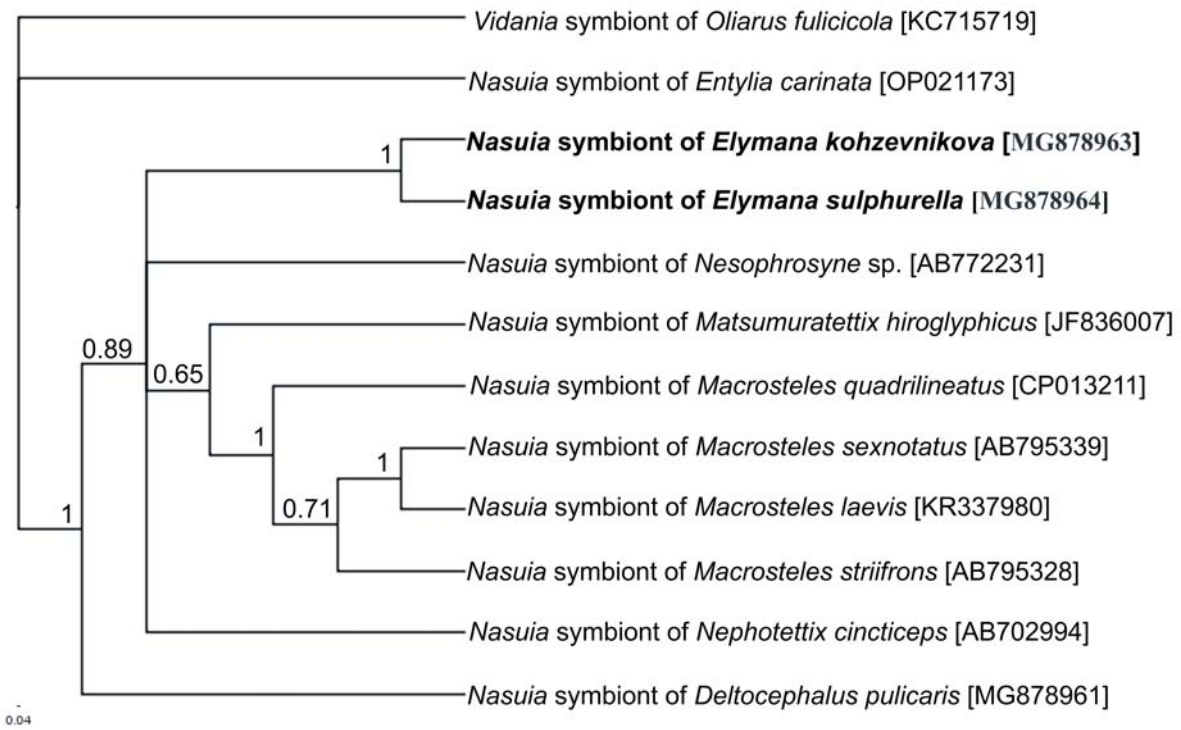
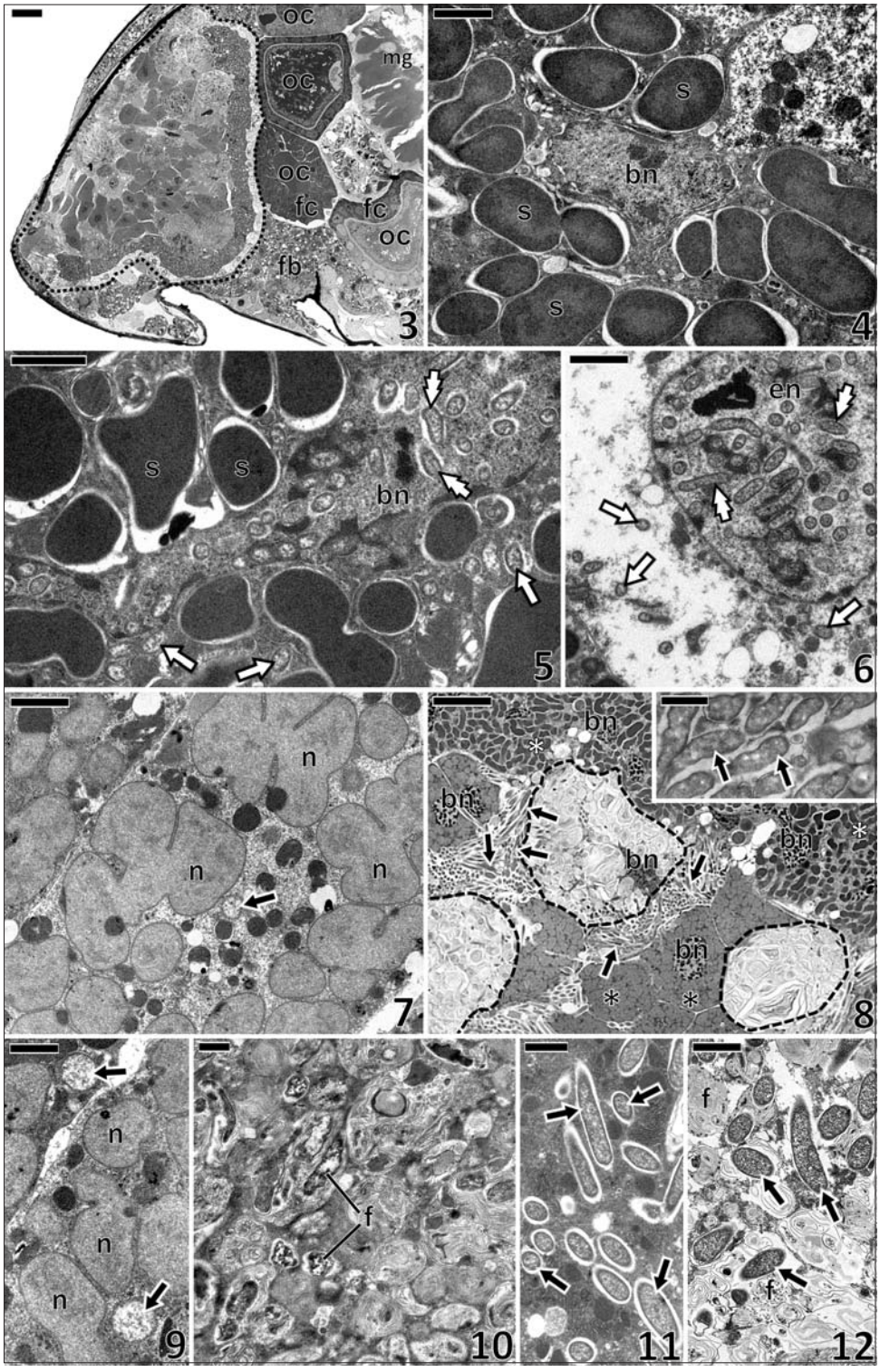


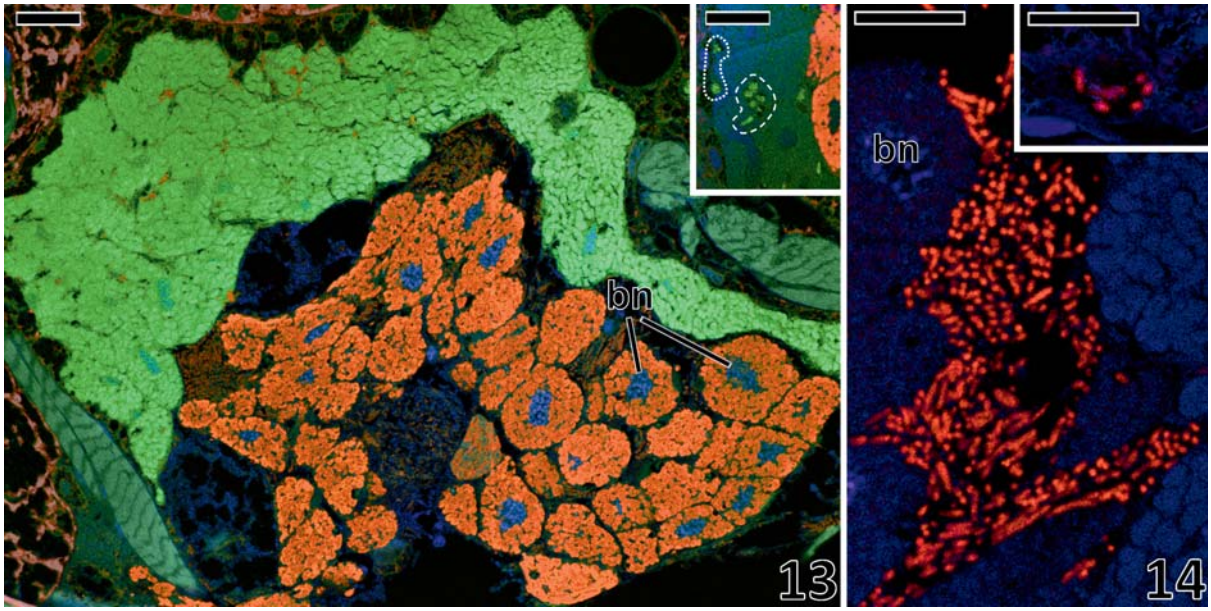
Fig. 2. Phylogenetic tree based on 16S rRNA sequences of *Nasuia* symbiont of the examined *Elymana kozhevnikovi and E. sulphurella* leafhoppers and other representatives of the subfamily Deltocephalinae, based on 16S RNA gene sequences. The numbers associated with the branches indicate Bayesian posterior probability values. The accession numbers of the sequences used in the phylogenetic analysis have been put in brackets. The *Vidania* symbiont of the planthopper *Oliarus fulicicola* was used as an outgroup.

Ultrastructure and distribution of symbiotic microorganisms

Histological observations revealed that paired bacteriomes occur in the females of *Elymana kozhevnikovi* and *E. sulphurella* (Fig. 3). Each bacteriome is composed of large bacteriocytes (Fig. 3). Two easily recognizable zones can be distinguished in the bacteriomes: a peripheral zone (Fig. 3) containing bacteriocytes with large, pleomorphic bacteria (Fig. 4) and a central zone (Fig. 3) with bacteriocytes containing large, lobated bacteria (Figs 7, 9) and large, elongated bacteria (Figs 7, 8, 8 insert, 9). Fluorescence *in situ* hybridization of the bacteriocyte-associated symbionts identified the pleomorphic microorganisms residing in peripheral bacteriocytes as *Sulcia* bacteria (Fig. 13), and the lobated microorganisms as *Nasuia* bacteria (Fig. 13). *Sulcia* stain more intensely with methylene blue (Figs 3, 8) and are more electron-dense under an electron transmission microscope (Figs 4, 5) compared to *Nasuia* (Figs 7, 9). In all the examined individuals of *E. kozhevnikovi* and *E. sulphurella*, in the cytoplasm and in the nuclei of some bacteriocytes with bacteria *Sulcia* (Fig. 5) and in some cells of the bacteriome sheath (Fig. 6) small, elongated, rod-shaped microorganisms occur. These microorganisms measure about 0.4 μm in diameter. FISH experiments using specific probes showed that *Sodalis*-like bacteria are present in bacteriocytes with *Sulcia* bacteria as well as in cells of the bacteriome sheath (Fig. 13 insert). In all the bacteriocytes with *Nasuia* large, elongated microorganisms also occur (Figs 7, 8, 8 insert, 9). The latter measure 1-1.2 μm in diameter. The use of the FISH technique identified these microorganisms as *Arsenophonus* bacteria (Fig. 14). It was observed that both in the younger females and in older (i.e. reproductive) females in some bacteriocytes *Arsenophonus* bacteria undergo degeneration (Figs 8, 10). In consequence, in these bacteriocytes numerous fagosomes and lamellar bodies appear (Fig. 10). Ultrastructural observations (Figs 11, 12) as well as FISH identification (Fig. 14 insert) revealed that both in *E. kozhevnikovi* and *E. sulphurella*, *Arsenophonus* bacteria are also present in fat body cells. Some *Arsenophonus* bacteria residing in fat body cells likewise undergo degeneration (Fig. 12).



Figs 3-12. Distribution of symbiotic bacteria in the body of *Elymana kozhevnikovi* and *E. sulphurella*. Fig. 3. *E. sulphurella*. Cross section through the abdomen. Note the large bacteriome localized ventrolaterally (marked with a black, dotted line). Fig. 4. *E. sulphurella*. Bacteriocyte with bacteria *Sulcia* (s). Fig. 5. *E. kozhevnikovi*. Bacteriocyte with bacteria *Sulcia* (s). Note small, elongated, rod-shaped bacteria in the bacteriocyte cytoplasm (white arrows) and in the nucleus (white, double arrows). Fig. 6. *E. kozhevnikovi*. Cell of the bacteriome sheath surrounding the bacteriome. Note small, elongated, rod-shaped bacteria in the bacteriocyte cytoplasm (white arrows) and in the nucleus (white, double arrows). Fig. 7. *E. kozhevnikovi*. Bacteriocyte with bacteria *Nasuia* (n). Note single bacterium *Arsenophonus* (black arrow) accompanying bacteria *Nasuia*. Fig. 8. *E. kozhevnikovi*. Fragment of the bacteriome containing bacteriocytes with bacteria *Sulcia* (white asterisks) and bacteriocytes with *Nasuia* (black asterisks) and *Arsenophonus* (black arrows). Note degenerating *Arsenophonus* bacteria (marked with a black, dashed line). Fig. 8 insert. *E. sulphurella*. *Arsenophonus* bacteria (black arrows). Fig. 9. *E. kozhevnikovi*. Fragment of two bacteriocytes with *Nasuia* (n). Note single *Arsenophonus* bacteria (black arrows) among *Nasuia*. Fig. 10. *E. sulphurella*. Fragment of bacteriocyte with *Nasuia* and *Arsenophonus*. Note fagosomes containing degenerating *Arsenophonus* bacteria (f). Figs 11, 12. *E. kozhevnikovi*. Fragment of fat body lobes with *Arsenophonus* bacteria. Note *Arsenophonus* (black arrows) and fagosomes (f). Figs 3, 8. LM, methylene blue, scale bar = 25µm. Figs 4–7, 7 insert, 9–12. TEM, scale bar =2µm. bn – bacteriocyte nucleus; en – nucleus of the cell of the bacteriome sheath; fb – fat body; fc – follicular epithelium; mg – midgut; oc – oocyte.



Figs 13-14. Fluorescence *in situ* identification of symbionts of *Elymana sulphurella*. Fig. 13. Bacteriocytes with *Sulcia* (shown in green) and *Nasuia* (shown in red) bacteria. Fig. 13 insert. *Sodalis*-like bacteria (shown in green) residing in bacteriocytes (marked with a white, dashed line) and in cells of the bacteriome sheath (marked with a white, dotted line). *Nasuia* is shown in red. Fig. 14. *Arsenophonus* bacteria residing in bacteriocytes (shown in red). Fig. 14 insert. *Arsenophonus* residing in fat body cells (shown in red). Confocal microscope, scale bar = 25μm. bn – bacteriocyte nucleus stained with DAPI.

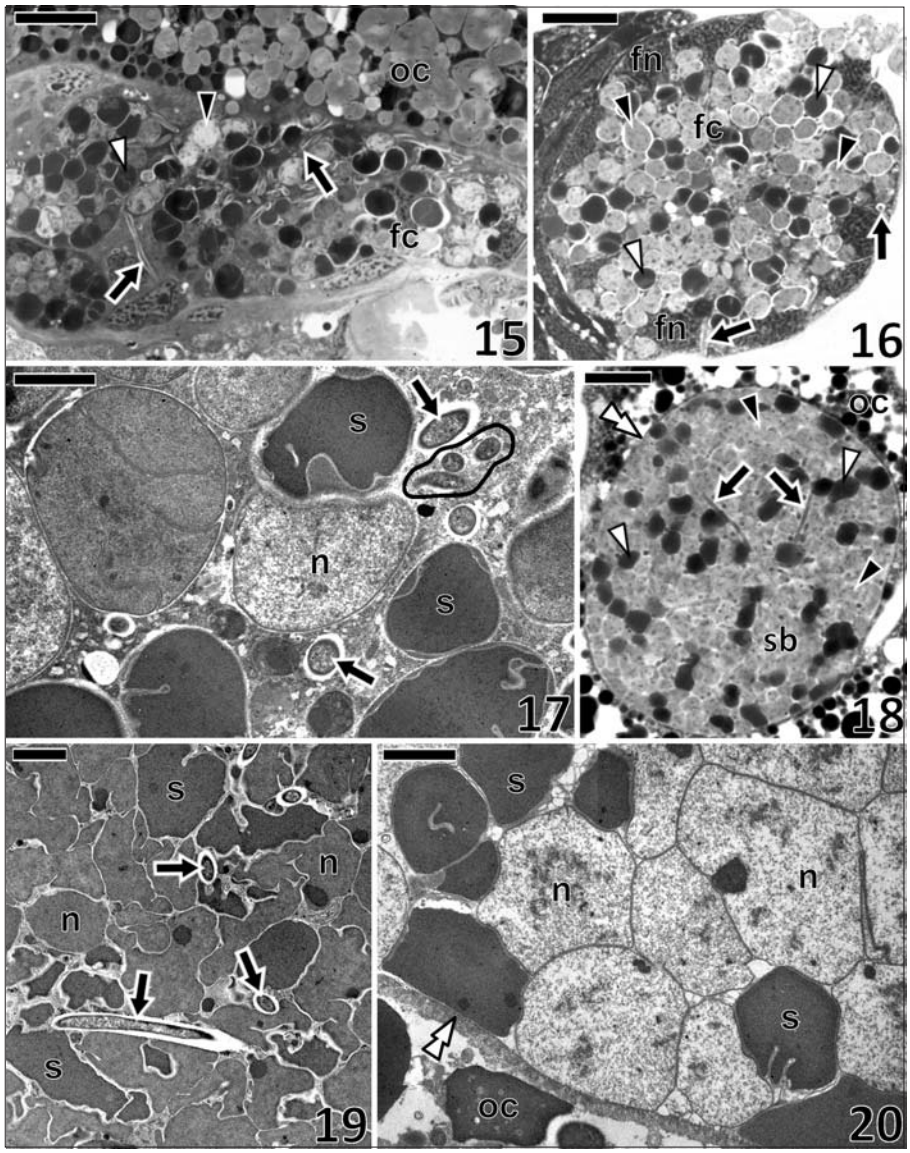
Transovarial transmission of symbionts

In reproductive females the bacteria leave the bacteriomes and invade ovaries. Ovaries of leafhoppers consist of seven elongated tubes called ovarioles. In each ovariole several linearly arranged oocytes are present. The oocytes are surrounded with a single layer of follicular cells (for further details concerning the organization of insect ovaries and course of oogenesis, see BÜNING 1994; BILIŃSKI 1998). The bacteria are released from the bacteriocytes and begin to invade follicular cells surrounding the posterior pole of the terminal oocytes which are at the stage of late vitellogenesis (Fig. 15). *Sulcia*, *Nasuia, Arsenophonus* and *Sodalis*-like bacteria enter the cytoplasm of follicular cells (Figs 16, 17). After passing through the follicular epithelium, bacteria accumulate in the space between the former and the oocyte surface (termed the perivitelline space), finally forming a "symbiont ball" (Fig. 18). The bacteria residing inside the "symbiont ball" closely adhere to each other (Figs 19, 20).

Discussion

Our morphological and molecular analyses revealed that in two species of Deltocephalinae leafhoppers, *Elymana kozhevnikovi* and *E. sulphurella*, an unusual combination of four microorganisms, *Sulcia*, *Nasuia*, *Arsenophonus* and *Sodalis*-like bacteria, occurs. To our knowledge, the co-existence of both ancient symbionts (i.e. *Sulcia* and betaproteobacteria) and more recently acquired *Arsenophonus* and *Sodalis*-like bacteria has not been observed in any other auchenorrhynchous hemipteran. Moreover, even the co-residence of three bacterial associates such as *Sulcia*, *Nasuia* and novel *Arsenophonus/Sodalis*-like bacteria is a very rare phenomenon within these insects. Both ancestral symbionts co-residing with *Arsenophonus* bacteria have only been found in the Deltocephalinae leafhopper *Macrosteles laevis* (KOBIAŁKA *et al*. 2016), whereas these symbionts co-residing with *Sodalis*-like bacteria have only been observed in the spittlebug, *Aphrophora quadrinotata* (Cicadomorpha, Cercopoidea: Aphrophoridae) (KOGA *et al*. 2013) and in the planthopper *Ommatidiotus dissimilis* (MICHALIK *et al*. 2018a). According to KOGA and co-workers (2013), the three-symbiont association in *A. quadrinotata* may be a transitional situation in which the novel symbiont *Sodalis* did not yet eliminate the ancestral betaproteobacterial symbiont *Zinderia*. The similarity in the organization of the symbiotic systems in *A. quadrinotata* and Deltocephalinae leafhoppers *E. kozhevnikovi* and *E. sulphurella* indicates a similar evolutionary scenario occurring in these hemipterans.

The situation observed in *E. kozhevnikovi* and *E. sulphurella* is of special interest. To our knowledge, the co-existence of *Nasuia* and *Arsenophonus* bacteria in the same bacteriocytes has never been reported for auchenorrhynchous hemipter-



Figs 15-20. Consecutive stages of transovarial transmission of symbiotic bacteria in *Elymana kozhevnikovi* and *E. sulphurella*. Fig. 15. *E. kozhevnikovi. Sulcia* (white arrowheads), *Nasuia* (black arrowheads) and *Arsenophonus* bacteria (black arrows) invade follicular cells surrounding the terminal (longitudinal section). Fig.16. *E. sulphurella.* Posterior pole of the ovariole (cross section). Follicular cells are tightly packed with symbiotic *Sulcia* (white arrowheads), *Nasuia* (black arrowheads) and *Arsenophonus bacteria* (black arrows) bacteria. Fig. 17. *E. kozhevnikovi. Sulcia* (s), *Nasuia* (n), *Arsenophonus* bacteria (black arrows) and small, rod-shaped *Sodalis*-like bacteria (marked with a black, continuous line) migrate through the cytoplasm of the follicular cell. Figs 18-20. "Symbiont ball" containing *Sulcia* (white arrowheads in LM images, s in TEM images), *Nasuia* (black arrowheads in LM images, n in TEM images) and *Arsenophonus* (black arrows) bacteria in a deep invagination of the oolemma (white, double arrowheads) at the posterior pole of the oocyte. Figs 18, 20. *E. sulphurella.* Fig. 19. *E. kozhevnikovi*. Figs 15, 16, 18. LM, methylene blue, scale bar =25μm. Figs 17, 19, 20. TEM, scale bar = 2μm. fc – follicular cell; fn – follicular cell nucleus; oc – oocyte; sb – symbiont ball.

ans. Moreover, ultrastructural observations clearly indicate that in all the examined females of *E. kozhevnikovi* and *E. sulphurella*, both in bacteriocytes and in fat body cells, numerous *Arsenophonus* bacteria undergo degeneration (see Figs 8, 10, 12). Based on morphological observations, it is very difficult to comment on this phenomenon. Additionally, it cannot be excluded that some of the lamellar bodies in the bacteriocytes may represent remnants of *Nasuia* bacteria. It may be speculated that *Arsenophonus*, as a novel symbiont of *E. kozhevnikovi* and *E. sulphurella*, may be neutralized by the host insect. This, in turn, corresponds well with the above-mentioned hypothesis of the intermediate state between the *Sulcia* and *Nasuia* system and the *Sulcia* and *Arsenophonus* system. Thus, in both of these leafhoppers, the ancestral *Nasuia* still exists and functions, but newly acquired *Arsenophonus* bacteria have already begun the long process of elimination of this symbiont. It seems that the occurrence of *Arsenophonus* both in the specialized bacteriocytes and in the fat body cells confirms the intermediate state of symbiosis in *Elymana*. The verification of this hypothesis will be the subject of further work with the use of genomic analyses.

Our PCR analyses revealed that *Sodalis*-like bacteria occur within all of the examined individuals of *E. kozhevnikovi* and *E. sulphurella*. The combination of results of ultrastructural observations and FISH method indicates that the microorganisms present in bacteriocytes with *Sulcia* and in the cells of the bacteriome sheath represent *Sodalis*-like bacteria. However, based on these results, we are unable to determine whether these bacteria are present both in the cytoplasm and in the cell nuclei. Taking into account the fact that *E. kozhevnikovi* and *E. sulphurella* are host to *Rickettsia* and *Wolbachia* which may occur intracellularly (ARNEODO *et al*. 2008; SCHULZ & HORN 2015; KOBIAŁKA in preparation), it cannot be excluded that *Sodalis*-like bacteria are distributed in the cytoplasm, whereas *Rickettsia* or *Wolbachia* may reside inside the nucleus. To resolve this question, further detailed studies are required. The role of *Sodalis*-like bacteria in the biology of *E. kozhevnikovi* and *E. sulphurella* remains unclear. Several facts such as: (1) the occurrence of these bacteria in all the examined individuals detected by means of ultrastructural and molecular methods, (2) their transovarial transmission between generations and (3) lack of any symptoms of negative influence on host insects, suggest that *Sodalis*-like bacteria may be beneficial to their host insects. This, in turn, leads to the conclusion that these bacteria may represent the most recently acquired symbionts.

Both *Arsenophonus* and *Sodalis*-like bacteria are widely distributed within insects (reviewed in DURON *et al.* 2008; NOVÁKOVÁ *et al*. 2009; WILKES *et al*. 2011). The interactions between *Arsenophonus* and insects may be parasitic or symbiotic (reviewed in WILKES *et al*. 2011). The symbiotic *Arsenophonus* (or its close relatives) has been found in hematophagous insects, for which it provides B vitamins (reviewed in WILKES *et al*. 2011). Within plant sap-feeding hemipterans, *Arsenophonus* has been detected in Cixiidae planthoppers, aphids, psyllids, whiteflies, the Deltocephalinae leafhopper *Macrosteles laevis* and the scale insect *Greenisca brachypodii* (WILKES *et al*. 2011; HALL *et al*. 2016; KOBIAŁKA *et al*. 2016; MICHALIK *et al*. 2018b). *Sodalis*-like symbionts have been observed, e.g. in tsetse flies (DALE & MAUDLIN 1999), heteropterans (KAIWA *et al*. 2010), scale insects (GATEHOUSE *et al*. 2011; KOGA *et al*. 2013; GRUWELL *et al*. 2014; HUSNIK & MCCUTCHEON 2016; SZKLARZEWICZ *et al*. 2018), leafhoppers (MICHALIK *et al*. 2014), spittlebugs (KOGA *et al.* 2013; KOGA & MORAN 2014), aphids (BURKE *et al*. 2009; MANZANO-MARIN *et al*. 2017), psyllids (THAO *et al*. 2000; HALL *et al*. 2016), phtirapterans (FUKATSU *et al*. 2007; BOYD *et al*. 2016), and weevils (TOJU & FUKATSU 2011; TOJU *et al*. 2013). Such a wide distribution of *Arsenophonus* and *Sodalis*-like symbionts among different groups of insects indicates the expansive nature of these bacteria and their tendency to replace the ancient symbionts.

Using PCR diagnostics we detected that apart from *Sulcia*, *Nasuia*, *Arsenophonus* and *Sodalis*-like symbionts, *E. kozhevnikovi* and *E. sulphurella* may harbor *Wolbachia* and *Rickettsia* bacteria widely distributed among arthropods. It seems probable that both these bacterial associates are dispersed in different tissues of these insects. However, to determine the detailed distribution of *Wolbachia* and *Rickettsia*, their role in the biology of the host insects and mode of transmission between generations, further comprehensive molecular and ultrastructural analyses are needed.

Our results provide new data on the symbionts of plant sap-sucking hemipterans and corroborate the previous observations that Deltocephalinae leafhoppers, uniquely among other hemipteran groups, are characterized by a large diversity of symbiotic systems. Numerous species of Deltocephalinae leafhoppers (*Deltocephalus pulicaris*, *Athysanus argentarius, Euscelis incisus, Doratura stylata, Arthaldeus pascuellus, Errastunus ocellaris, Jassargus flori, Jassargus pseudocellaris, Psammotettix alienus, Psammotettix confinis, Turrutus socialis and Verdanus abdominalis, Macrosteles quadripunctatus*, *Macrosteles quadrilineatus*, *Macrosteles sexnotatus, Macrosteles striifrons, Matsumuratettix hiroglyphicus*, *Nephotettix cincticeps*) retained the ancient symbionts, i.e. *Sulcia* and *Nasuia* (WANGKEEREE *et al*. 2011; NODA *et al*. 2012; BENNETT & MORAN 2013; ISHII *et al.* 2013; KOBIAŁKA *et al.* 2015; BENNETT *et al.* 2016; KOBIAŁKA *et al*. in preparation). In *E. kozhevnikovi* and *E. sulphurella*, apart from ancient symbionts, novel symbionts – *Arsenophonus* and *Sodalis*-like bacteria are present (this study), whereas in *Macrosteles laevis* – the ancient symbionts are accompanied by *Arsenophonus* (KOBIAŁKA *et al.* 2016). It should be stressed that in *M. laevis*, the bacterium *Arsenophonus* does not occur individually, but is hidden inside cells of *Sulcia* bacteria. In *Cicadula quadrinotata*, *Fieberiella septentrionalis*, *Graphocraerus ventralis* and *Orientus ishidae*, the bacterium *Nasuia* has been lost and replaced by yeast-like symbionts (KOBIAŁKA *et al.* 2017), whereas in *Balclutha calamagrostis* and *Balclutha punctata* – by *Sodalis*-like bacteria (KOBIAŁKA *et al*. in preparation). In *Dalbulus maidis*, in turn, only *Sulcia* has been detected (BRENTASSI *et al.* 2017). These results indicate that ancient symbionts of Deltocephalinae leafhoppers undergo elimination and replacement by novel symbionts, such as *Arsenophonus*, *Sodalis* and yeast-like microorganisms.

Observations of the course of transmission of symbionts from the mother to offspring in different species of Deltocephalinae leafhoppers indicate that this process is uniform in this group of hemipterans (MÜLLER 1962; BUCHNER 1965; KOBIAŁKA *et al.*

2015, 2016, 2017; BRENTASSI *et al.* 2017). In all the hitherto examined Deltocephalinae leafhoppers, the symbiotic microorganisms (both the ancient symbionts and novel associates – bacteria or yeast-like symbionts) invade the posterior pole of the ovariole. The symbionts individually migrate through the cytoplasm of follicular cells surrounding the terminal oocytes and next they gather in the perivitelline space in the deep invagination of oolemma in the form of a "symbiont ball". The single exception is *M. laevis* in which the novel associate *Arsenophonus* does not infect the ovarioles individually, but is transported inside cells of *Sulcia*. Thus, this atypical behavior of *Arsenophonus* is probably connected with the young condition of association between this microorganism and *M. laevis*. Our ultrastructural observations have shown that in *E. kozhevnikovi* and *E. sulphurella, Arsenophonus* and *Sodalis*-like bacteria are individually transmitted to the ovariole. This, in turn, indicates that both species of *Elymana* have already developed a stable system of novel symbiont transmission.

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Author contributions

Research concept and design: M.K., T.S.; Collection and/or assembly of data: M.K.; Data analysis and interpretation: M.K., A.M., T.S.; Writing the article: M.K., A.M., T.S.; Critical revision of the article: T.S.; Final approval of article: T.S.

Conflict of Interest

The authors declare no conflict of interest.

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