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Infection by Endosymbiotic "Male-Killing" Bacteria in Coleoptera

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Original article

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Wolbachia, *Rickettsia*, *Spiroplasma* and *Cardinium* are endosymbiotic and intracellular bacteria known to cause numerous disorders in host reproduction, reflected in their common name "male-killers". In this study, 297 beetle species from various taxonomic groups were screened with the use of molecular markers for the presence of infection by any of these endosymbionts. *Wolbachia* was found to be the most common "male-killer" among beetle hosts as it infected approx. 27% of species. *Rickettsia*, *Spiroplasma* and *Cardinium* were much less prevalent as they infected: 8%, 3% and 2%, respectively, of the studied beetle species. This is the first report of *Cardinium* presence in beetle hosts. Incidences of co-infection of two bacteria taxa were very rare and only two weevil species were found to be infected by three different bacteria. These findings suggest that endosymbiotic bacteria inhabit their host at various levels of prevalence and that particular taxa usually infect different hosts, suggesting some competition among "male-killers". This is the first study that simultaneously verifies infection status by all major endosymbiotic bacteria in hundreds of species (based on coleopterans).

Key words: *Wolbachia*, *Rickettsia*, *Spiroplasma*, *Cardinium*, beetle, intracellular infection.

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Evolution of mutualistic symbioses between unicellular and multicellular organisms was key to starting a new era for many life forms. Not only did it accelerate the evolution of eukaryotic organisms, but most herbivores would not be able to digest without their symbionts (MARGULIS & FESTER 1991). Insects are hosts to a wide range of microorganisms called endosymbionts that live within the host body or cells (MARGULIS & CHAPMAN 2009). The relationship between host and endosymbiont vary from mutualistic (the host derives a fitness benefit) to parasitic (the host suffers a decline in fitness), whereas the majority of bacteria are probably commensals (BUCHNER 1965; ITURBE-ORMAETXE & O'NEILL 2007). The specific group of endosymbionts are so called "male-killing" bacteria. The best known are two genera of α-proteobacteria: *Wolbachia* (Anaplasmataceae) and *Rickettsia* (Rickettsiaceae), others like *Spiroplasma* (Tenericutes) and *Cardinium* (Bacteroidetes) are much less studied (BOVÉ 1997; CASPI-FLUGER *et al.* 2011; KAJTOCH & KOTÁSKOVÁ

2018; ZCHORI-FEIN & PERLMAN 2004). *Wolbachia* has been reported from arthropods and filarial nematodes around the world (TAYLOR & HOERAUF 1999; WERREN & WINDSOR 2000), also *Rickettsia* seemed to be a common bacterial symbiont of arthropods (PERLMAN *et al.* 2006). In arthropods, they can manipulate host reproduction through „male-killing" (JIGGINS *et al.* 2001; LAWSON *et al.* 2001), cytoplasmic incompatibility (POINSOT *et al.* 2003), parthenogenesis induction (HAGIMORI *et al.* 2006; STOUTHAMER *et al.* 1999) and feminization of genetic males (HIROKI *et al.* 2002). These effects on host reproduction and development could result in diversification of populations and consequently lead to speciation. On the other hand, many species naturally infected by „male-killing" bacteria (e.g. *Drosophila melanogaster*; HOFFMANN *et al.* 1998) do not manifest disruption in sex ratio, while the endosymbiont influences other host traits such as: increasing insulin signaling levels (IKEYA *et al.* 2009), female fecundity (FRY *et al.* 2004; FRY & RAND 2002) or inducing host resistance to viral infections (TEIXEIRA *et al.* 2008) and confers protection against wasp parasitism (XIE *et al.* 2010). Moreover *Wolbachia* may be an obligatory bacteriocyte-associated nutritional mutualist (HOSOKAWA *et al.* 2010), highlighting a previously unknown aspect of the parasitism-mutualism evolutionary continuum.

Beetles (Coleoptera) are the most species-rich and diversified order of insects in the world, including approximately 386,000 known species (ŚLIPIŃSKI *et al.* 2011). Knowledge about endosymbiont prevalence within this group is poorly established. Only a few studies so far have found *Wolbachia* with *Rickettsia* and/or *Spiroplasma* together in beetle hosts (BILI *et al.* 2016; DUDEK *et al.* 2017; MAJERUS *et al.* 2000; PEROTTI *et al.* 2016; SCHEBECK *et al.* 2018; TOJU & FUKATSU 2011; WEINERT *et* al. 2007; WHITE *et al.* 2015). *Wolbachia* is the best studied organism in this case, with approx. 38% of infection among beetles (KAJTOCH & KOTÁSKOVÁ 2018), next *Rickettsia* being mostly screened among ladybird beetles (WEINERT *et al.* 2009) and on single species: the buprestid leaf-mining beetle *Brachys tessellates* (LAWSON *et al.* 2001) and bruchid beetle *Kytorhinus sharpianus* (FUKATSU & SHIMADA 1999). *Spiroplasma* has been studied mostly on single beetle species, e.g. the ladybird beetle *Anisosticta novemdecimpunctata* (TINSLEY & MAJERUS 2006), leaf beetles *Leptinotarsa decemlineata* (CLARK 1982) and *Diabrotica undecimpunctata*, lampyrid beetle *Ellychnia corrusca*, cantharid beetles *Cantharis bilineatus* and *Cantharis carolinus* (CLARK *et al.* 1987), bark beetle *Pityogenes chalcographus* (SCHEBECK *et al.* 2018). Surprisingly it seems that *Cardinium* has never been found within beetles (SCHEBECK *et al.* 2018; ZCHORI-FEIN & PERLMAN 2004).

TOJU & FUKATSU (2011) showed that pairs of some endosymbionts (e.g. *Wolbachia–Rickettsia* and *Rickettsia–Spiroplasma*) infected the same host more frequently than others. Although interesting, their study focused only on Japanese populations of chestnut weevil *Curculio sikkimensis* (Curculionidae). On the other hand GOTO *et al*. (2006) suggested that there is some balance in the number of these bacteria, probably caused by competition within host cells. The complexity of endosymbiont interactions and their effects on their hosts may play a pivotal role for diversification of host populations and consequently on speciation (e.g., by the selective sweep of mtDNA or the whole genome of the infected host with the genome of bacteria; KELLER *et al*. 2004; MAZUR *et al.* 2016). Consequently, it was proved that presence of endosymbiotic "male-killers" could bias species identification via barcoding (SMITH *et al.* 2012).

This study, by verifying infection status of *Wolbachia*, *Rickettsia*, *Spiroplasma* and *Cardinium* across 297 beetle taxa, aimed to answer the question of prevalence of endosymbionts within their beetle hosts. Moreover, co-occurrence of these bacteria in particular beetle species was analyzed in respect to their potential mutually exclusive distribution within the hosts.

Material and Methods

Sampling

Beetle species were collected during several field trips organized across central, eastern, and southeastern Europe from 2014 to 2017. Beetle species examined in this study were mostly collected in Poland, Slovakia, Romania, and Bulgaria; some taxa were also taken from Czechia, Germany, Austria, Hungary, Croatia, Greece, Ukraine, and Belarus (192 sites in total, Suppl. Table 1). Beetles were caught in various habitats using numerous entomological techniques (using sweep-net, sieve, light-capturing, traps for scavengers, searching in dead wood, mushrooms, etc.) by experienced entomologists – specialists in various groups of beetles. After collection specimens were immediately preserved in 96% ethanol and afterwards deposited in laboratories in -20ºC. Next, taxonomists assigned the collected beetles to species level. The nomenclature adopted in this study follows that of the Catalogue of Palaearctic Coleoptera (LÖBL & SMETANA 2003-2013). Finally 297 species were selected for molecular examination. The majority of these species were analyzed using two to five or more specimens; only some (mainly rare taxa) could be examined using a single representative. These beetles belonged to 37 families and 204 genera.

Laboratory methods

Before DNA extraction all specimens were cleaned using ethanol and distilled water in order to reduce the risk of external contamination. DNA was extracted from the whole insect body (for beetles up to approximately 10 mm length; only from abdomen for larger specimens) using the Nucleospin Tissue kit (Macherey-Nagel), following the manufacturer’s instructions. Prior to bacteria screening, the quality of DNA isolates was assessed by amplification cytochrome oxidase, subunit I of mtDNA (with use of primers specific for beetles from FOLMER *et al.* 1994; HEBERT *et al.* 2003) and for almost all isolates amplification of this gene was successful (see Suppl. Table 1). The

Table 1

Taxonomy and infection status of examined beetle species

No.	Species	Family	No of examined individuals	*Wolbachia*	*Rickettsia*	*Spiroplasma*	*Cardinium*
1	*Abax parallelepipedus*	Carabidae	2				
2	*Abax parallelus*	Carabidae	1	✓			
3	*Adalia bipunctata*	Coccinellidae	2				
4	*Agapanthia villosoviridescens*	Cerambycidae	3				
5	*Agonum marginatum*	Carabidae	3	✓			
6	*Agrilus derasofasciatus*	Buprestidae	1				
7	*Agrilus suvorovi*	Buprestidae	2				
8	*Agrypnus murinus*	Elateridae	1				
9	*Alosterna tabacicolor*	Cerambycidae	3				
10	*Altica oleracea*	Chrysomelidae	3				✓
11	*Amara similata*	Carabidae	1	✓			
12	*Amphimallon solstitialis*	Scarabaeidae	2				
13	*Anaspis brunnipes*	Scraptiidae	3	✓			
14	*Anaspis frontalis*	Scraptiidae	4	✓			
15	*Anastrangalia reyi*	Cerambycidae	2	✓			
16	*Anastrangalia sanguinolenta*	Cerambycidae	2				
17	*Anatis ocellata*	Coccinellidae	1				
18	*Anomala dubia*	Scarabaeidae	3				
19	*Anoplodera sexguttata*	Cerambycidae	3				
20	*Anoplotrupes stercorosus*	Geotrupidae	3				
21	*Anostirus castaneus*	Elateridae	2				
22	*Anthaxia nitidula*	Buprestidae	3		✓		
23	*Anthaxia quadripunctata*	Buprestidae	2				
24	*Anthonomus rubi*	Curculionidae	3	✓			
25	*Aphodius ater*	Scarabaeidae	2				
26	*Aphodius depressus*	Scarabaeidae	5				
27	*Aphodius erraticus*	Scarabaeidae	3				
28	*Aphodius fossor*	Scarabaeidae	2				
29	*Aphodius granarius*	Scarabaeidae	3	✓			
30	*Aphodius haemorrhoidalis*	Scarabaeidae	3	✓			
31	*Aphodius luridus*	Scarabaeidae	3				
32	*Aphodius pedellus*	Scarabaeidae	3				
33	*Aphodius pusillus*	Scarabaeidae	3				
34	*Aphodius scrutator*	Scarabaeidae	2				
35	*Aphodius sphacelatus*	Scarabaeidae	3				
36	*Aphodius sticticus*	Scarabaeidae	3	✓			
37	*Aphthona venustula*	Chrysomelidae	3	✓	✓		
38	*Arhopalus rusticus*	Cerambycidae	2	✓			
39	*Aromia moschata*	Cerambycidae	1				
40	*Athous subfuscus*	Elateridae	1				

Table 1 cont.

No.	Species	Family	No of examined individuals	*Wolbachia*	*Rickettsia*	*Spiroplasma*	*Cardinium*
41	*Attagenus pellio*	Dermestidae	1				
42	*Batophila rubi*	Chrysomelidae	3	✓			
43	*Bembidion articulatum*	Carabidae	3		✓		
44	*Bembidion decorum*	Carabidae	3				
45	*Bembidion modestum*	Carabidae	3				
46	*Bembidion punctulatum*	Carabidae	4	✓			
47	*Bembidion varicolor*	Carabidae	6	✓			
48	*Bembidion varium*	Carabidae	3	✓			
49	*Bitoma crenata*	Zopheridae	3				
50	*Blitopertha lineolata*	Scarabaeidae	3				
51	*Bostrichus capucinus*	Bostrichidae	2				
52	*Brachyleptura maculicornis*	Cerambycidae	2				
53	*Broscus cephalotes*	Carabidae	2				
54	*Bruchidius cf. varius*	Chrysomelidae	3	✓			
55	*Bruchus affinis*	Chrysomelidae	3				
56	*Bruchus atomarius*	Chrysomelidae	3	✓			
57	*Bruchus brachialis*	Chrysomelidae	3				
58	*Bruchus loti*	Chrysomelidae	3				
59	*Bruchus viciae*	Chrysomelidae	3				
60	*Caccobius schreberi*	Scarabaeidae	2				
61	*Calathus fuscipes*	Carabidae	3				
62	*Calvia quatuordecimpunctata*	Coccinellidae	2				
63	*Cantharis livida*	Cantharidae	2				
64	*Cantharis nigricans*	Cantharidae	3				
65	*Cantharis rustica*	Cantharidae	2			✓	
66	*Carabus cancellatus*	Carabidae	2				
67	*Carabus coriaceus*	Carabidae	4				
68	*Carabus glabratus*	Carabidae	3	✓			
69	*Carabus granulatus*	Carabidae	4				
70	*Carabus hortensis*	Carabidae	4				
71	*Carabus nemoralis*	Carabidae	4				
72	*Carabus violaceus*	Carabidae	2				
73	*Cassida viridis*	Chrysomelidae	3	✓			✓
74	*Catapion jaffense*	Apionidae	2	✓			
75	*Catapion koestlini*	Apionidae	2				
76	*Catapion meieri*	Apionidae	2	✓			
77	*Catapion pubescens*	Apionidae	2				
78	*Catapion seniculus*	Apionidae	2	✓			
79	*Centricnemus leucogrammus*	Curculionidae	3				
80	*Cetonia aurata*	Scarabaeidae	4				
81	*Ceutorhynchus obstrictus*	Curculionidae	6	✓			
82	*Ceutorhynchus typhae*	Curculionidae	3	✓			
83	*Cheilotoma musciformis*	Chrysomelidae	3				
84	*Chilocorus renipustulatus*	Coccinellidae	2				
85	*Chlaenius nitidulus*	Carabidae	1				
86	*Chlaenius tibialis*	Carabidae	3				
87	*Chrysanthia geniculata*	Oedemeridae	2				
88	*Chrysolina herbacea*	Chrysomelidae	3	✓			
89	*Chrysolina polita*	Chrysomelidae	3			✓	
90	*Chrysolina varians*	Chrysomelidae	3			✓	
91	*Cicindela hybrida*	Carabidae	3				
92	*Clerus mutillarius*	Cleridae	3				
93	*Clivina collaris*	Carabidae	1				

Table 1 cont.

No.	Species	Family	No of examined individuals	*Wolbachia*	*Rickettsia*	*Spiroplasma*	*Cardinium*
95	*Coccinella septempunctata*	Coccinellidae	4				
96	*Coccinula quatuordecimpustulata*	Coccinellidae	3	✓			
97	*Combocerus glaber*	Erotylidae	1	✓			
98	*Coraebus elatus*	Buprestidae	4				
99	*Crepidodera aurata*	Chrysomelidae	3	✓			
100	*Crioceris asparagi*	Chrysomelidae	3				
101	*Crioceris quatuordecimpunctata*	Chrysomelidae	4	✓			
102	*Crioceris quinquepunctata*	Chrysomelidae	4	✓			
103	*Cryptocephalus bameuli*	Chrysomelidae	3				
104	*Cryptocephalus bipunctatus*	Chrysomelidae	3				
105	*Cryptocephalus flavipes*	Chrysomelidae	3				
106	*Cryptocephalus hypochoeridis*	Chrysomelidae	2	✓			
107	*Cryptocephalus moraei*	Chrysomelidae	3				
108	*Cryptocephalus quadripustulatus*	Chrysomelidae	1				
109	*Cryptocephalus sericeus*	Chrysomelidae	3				
110	*Cryptocephalus signatifrons*	Chrysomelidae	1				
111	*Cryptocephalus violaceus*	Chrysomelidae	2				
112	*Cyanapion afer*	Apionidae	3	✓			
113	*Cyanapion gnarum*	Apionidae	3				
114	*Cyanapion gyllenhalii*	Apionidae	3	✓			
115	*Cyanapion platalea*	Apionidae	3				
116	*Cyanapion columbinum*	Apionidae	3				
117	*Cyanapion spencii*	Apionidae	3	✓			
118	*Dacne bipustulata*	Erotylidae	1				
119	*Dacne rufifrons*	Erotylidae	3	✓			
120	*Dalopius marginatus*	Elateridae	1				
121	*Dascillus cervinus*	Dascillidae	2				
122	*Dasytes plumbeus*	Dasytidae	3		✓		
123	*Deporaus betulae*	Rhynchitidae	2				
124	*Dermestes lardarius*	Dermestidae	2				
125	*Derocrepis rufipes*	Chrysomelidae	3		✓		
126	*Diaperis boleti*	Tenebrionidae	2				
127	*Dinoptera collaris*	Cerambycidae	3		✓		
128	*Dolichosoma lineare*	Dasytidae	2		✓		
129	*Dorcus parallelipipedus*	Lucanidae	3				
130	*Dromius agilis*	Carabidae	1	✓			
131	*Dryocoetes autographus*	Curculionidae	3				
132	*Drypta dentata*	Carabidae	1				
133	*Dyschirius digitatus*	Carabidae	2				
134	*Elaphropus quadrisignatus*	Carabidae	2				
135	*Elaphrus aureus*	Carabidae	1				
136	*Elaphrus riparius*	Carabidae	3				
137	*Etorofus pubescens*	Cerambycidae	2	✓			
138	*Euoniticellus fulvus*	Scarabaeidae	3				
139	*Eusomus ovulum*	Curculionidae	5	✓			
140	*Eutrichapion melancholicum*	Apionidae	2				
141	*Eutrichapion viciae*	Apionidae	2				
142	*Exochomus quadripustulatus*	Coccinellidae	4				
143	*Galeruca tanaceti*	Chrysomelidae	3				
144	*Galerucella tenella*	Chrysomelidae	3				
145	*Gnaptor spinimanus*	Tenebrionidae	4				
146	*Harpalus affinis*	Carabidae	2				
147	*Harpalus rufipes*	Carabidae	3				

Table 1 cont.

No.	Species	Family	No of examined individuals	*Wolbachia*	*Rickettsia*	*Spiroplasma*	*Cardinium*
149	*Hylobius abietis*	Curculionidae	3	✓			
150	*Hylotrupes bajulus*	Cerambycidae	2				
151	*Ips typographus*	Curculionidae	3				
152	*Kateretes pedicularius*	Nitidulidae	3				
153	*Lagria hirta*	Tenebrionidae	3	✓			
154	*Litargus connexus*	Mycetophagidae	1				
155	*Lixus filiformis*	Curculionidae	3				
156	*Lochmaea caprea*	Chrysomelidae	3				
157	*Longitarsus exsoletus*	Chrysomelidae	3				
158	*Longitarsus nasturtii*	Chrysomelidae	3				
159	*Loricera pilicornis*	Carabidae	3				
160	*Luperus flavipes*	Chrysomelidae	3	✓			
161	*Luperus luperus*	Chrysomelidae	3	✓	✓		
162	*Lygistopterus sanguineus*	Lycidae	3				
163	*Mecinus pascuorum*	Curculionidae	3	✓	✓		
164	*Melolontha melolontha*	Scarabaeidae	3				
165	*Metacantharis discoidea*	Cantharidae	3				
166	*Monochamus galloprovincialis*	Cerambycidae	2				
167	*Monochamus sartor*	Cerambycidae	3	✓			
168	*Monochamus sutor*	Cerambycidae	2				
169	*Mordella brachyura*	Mordellidae	3				
170	*Mordella holomelaena*	Mordellidae	3				
171	*Musaria affinis*	Cerambycidae	3				
172	*Mycetophagus ater*	Mycetophagidae	1				
173	*Mycetophagus multipunctatus*	Mycetophagidae	1				
174	*Mycetophagus piceus*	Mycetophagidae	1				
175	*Mycetophagus quadripustulatus*	Mycetophagidae	2				
176	*Myrrha octodecimguttata*	Coccinellidae	3	✓			
177	*Neatus picipes*	Tenebrionidae	1				
178	*Nebria picicornis*	Carabidae	1	✓			
179	*Neocoenorhinus germanicus*	Rhynchitidae	3	✓			
180	*Nedyus quadrimaculatus*	Curculionidae	3	✓			
181	*Nicrophorus vespilloides*	Silphidae	3	✓			
182	*Nivellia sanguinosa*	Cerambycidae	3		✓		
183	*Notiophilus biguttatus*	Carabidae	2				
184	*Notoxus monoceros*	Anthicidae	3	✓			
185	*Obrium brunneum*	Cerambycidae	1		✓		
186	*Ocypus olens*	Staphylinidae	1				
187	*Oedemera femorata*	Oedemeridae	3				
188	*Oedemera flavipes*	Oedemeridae	3				
189	*Oenopia conglobata*	Coccinellidae	1				
190	*Oiceoptoma thoracica*	Silphidae	1	✓			
191	*Omalisus fontisbellaquaei*	Omalisidae	3				
192	*Omonadus floralis*	Anthicidae	1				
193	*Omophron limbatum*	Carabidae	2				
194	*Onthophagus fracticornis*	Scarabaeidae	3	✓			
195	*Onthophagus ovatus*	Scarabaeidae	3				
196	*Onthophagus ruficapillus*	Scarabaeidae	6	✓			
197	*Onthophagus similis*	Scarabaeidae	4	✓			
198	*Onthophagus taurus*	Scarabaeidae	8	✓			
199	*Ophonus laticollis*	Carabidae	1				
200	*Otiorhynchus perdix*	Curculionidae	3		✓		
201	*Otiorhynchus riessi*	Curculionidae	3		✓		
202	*Oulema gallaeciana*	Chrysomelidae	3				

Table 1 cont.

No.	Species	Family	No of examined individuals	*Wolbachia*	*Rickettsia*	*Spiroplasma*	*Cardinium*
203	*Oxymirus cursor*	Cerambycidae	1				
204	*Oxythyrea funesta*	Scarabaeidae	3			✓	
205	*Pachyta quadrimaculata*	Cerambycidae	2	✓			
206	*Pachytodes cerambyciformis*	Cerambycidae	2				
207	*Paederidus rubrothoracicus*	Staphylinidae	4				
208	*Paederidus ruficollis*	Staphylinidae	2	✓			
209	*Paederus caligatus*	Staphylinidae	1			✓	
210	*Paederus limnophilus*	Staphylinidae	4	✓		✓	
211	*Paederus litoralis*	Staphylinidae	1	✓			
212	*Paederus riparius*	Staphylinidae	3	✓			
213	*Paophilus afflatus*	Curculionidae	1	✓			
214	*Paradromius linearis*	Carabidae	2				
215	*Parafoucartia squamulata*	Curculionidae	1	✓			
216	*Paranchus albipes*	Carabidae	1				
217	*Perileptus areolatus*	Carabidae	2				
218	*Phosphuga atrata*	Silphidae	2				
219	*Phyllobius brevis*	Curculionidae	3				✓
220	*Phyllobius glaucus*	Curculionidae	3	✓	✓		
221	*Phyllobius maculicornis*	Curculionidae	3	✓			
222	*Phyllopertha horticola*	Scarabaeidae	3				
223	*Phyllotreta christinae*	Chrysomelidae	2				
224	*Phymatodes testaceus*	Cerambycidae	2				
225	*Pidonia lurida*	Cerambycidae	3		✓		
226	*Pityogenes chalcographus*	Curculionidae	3	✓			
227	*Plagionotus detritus*	Cerambycidae	2				
228	*Platynus assimilis*	Carabidae	3				
229	*Poecilus versicolor*	Carabidae	1				
230	*Polydrusus mollis*	Curculionidae	3	✓			
231	*Polydrusus inustus*	Curculionidae	2	✓	✓	✓	
232	*Prionus coriarius*	Cerambycidae	3				
233	*Propylea quatuordecimpunctata*	Coccinellidae	3				
234	*Pseudomechoris aethiops*	Rhynchitidae	3				✓
235	*Pseudoprotapion ergenense*	Apionidae	3	✓	✓		
236	*Pseudovadonia livida*	Cerambycidae	1				
237	*Psyllobora vigintiduopunctata*	Coccinellidae	2	✓		✓	
238	*Pterostichus melanarius*	Carabidae	3				
239	*Pterostichus niger*	Carabidae	4				
240	*Pterostichus oblongopunctatus*	Carabidae	4				
241	*Pyrochroa coccinea*	Pyrochroidae	2				
242	*Pyrrhidium sanguineum*	Cerambycidae	3		✓		
243	*Pytho depressus*	Pythidae	2				
244	*Rhagium inquisitor*	Cerambycidae	2				
245	*Rhagium mordax*	Cerambycidae	2				
246	*Rhagium sycophanta*	Cerambycidae	2				
247	*Rhagonycha fulva*	Cantharidae	2				
248	*Rhagonycha lignosa*	Cantharidae	2				
249	*Rhagonycha limbata*	Cantharidae	3				
250	*Rhinusa tetra*	Curculionidae	3	✓	✓		
251	*Rhyzobius chrysomeloides*	Coccinellidae	2		✓		
252	*Rusticoclytus rusticus*	Cerambycidae	3				
253	*Rutpela maculata*	Cerambycidae	1				
254	*Salpingus ruficollis*	Salpingidae	1				
255	*Saperda scalaris*	Cerambycidae	2				
256	*Schizotus pectinicornis*	Pyrochroidae	3				

Table 1 cont.

No.	Species	Family	No of examined individuals	*Wolbachia*	*Rickettsia*	*Spiroplasma*	*Cardinium*
257	*Sciaphobus rubi*	Curculionidae	1	✓			
258	*Scymnus nigrinus*	Coccinellidae	1				
259	*Serropalpus barbatus*	Melandryidae	2				
260	*Sibinia pellucens*	Curculionidae	3	✓			✓
261	*Silis nitidula*	Cantharidae	3				
262	*Sitona suturalis*	Curculionidae	3	✓	✓		
263	*Sitona sulcifrons*	Curculionidae	3				
264	*Smaragdina affinis*	Chrysomelidae	1				
265	*Spermophagus sericeus*	Chrysomelidae	3				
266	*Sphindus dubius*	Sphindidae	2				
267	*Spondylis buprestoides*	Cerambycidae	2				
268	*Staphylinus erythropterus*	Staphylinidae	2	✓			
269	*Stenolophus teutonus*	Carabidae	1				
270	*Stenomax aeneus*	Tenebrionidae	3	✓			
271	*Stenurella melanura*	Cerambycidae	2				
272	*Stenurella nigra*	Cerambycidae	4				
273	*Strangalia attenuata*	Cerambycidae	2				
274	*Strophosoma capitatum*	Curculionidae	4	✓			
275	*Strophosoma faber*	Curculionidae	1				
276	*Strophosoma melanogrammum*	Curculionidae	5	✓			
277	*Synapion ebeninum*	Apionidae	2				
278	*Synuchus vivalis*	Carabidae	1				
279	*Tachyta nana*	Carabidae	3				
280	*Tanymecus palliatus*	Curculionidae	3	✓		✓	✓
281	*Tenebrio molitor*	Tenebrionidae	3				
282	*Tetropium castaneum*	Cerambycidae	3				
283	*Tetropium fuscum*	Cerambycidae	2	✓			
284	*Thanasimus formicarius*	Cleridae	2				
285	*Thanatophilus sinuatus*	Silphidae	2				
286	*Trachys minuta*	Buprestidae	3	✓			
287	*Trichodes apiarius*	Cleridae	3				
288	*Triplax aenea*	Erotylidae	1	✓			
289	*Triplax russica*	Erotylidae	1				
290	*Tritoma bipustulata*	Erotylidae	2				
291	*Tropinota hirta*	Scarabaeidae	3				
292	*Trypocopris vernalis*	Geotrupidae	3				
293	*Trypodendron lineatum*	Curculionidae	3				
294	*Tytthaspis sedecimpunctata*	Coccinellidae	1				
295	*Uleiota planata*	Silvanidae	3				
296	*Uloma culinaris*	Tenebrionidae	3	✓			
297	*Valgus hemipterus*	Scarabaeidae	3				

presence of *Wolbachia* in particular beetles was first screened using two sets of primers amplifying *Wolbachia* surface protein (wsp) and cell division protein (ftsZ) (primers and PCR conditions follow https://pubmlst.org/*Wolbachia*/). Two controls were used in this step: negative (samples with distilled water instead of DNA isolates) and positive (DNA isolates from *Polydrusus inustus* weevil, which is known to be infected in its entire range). Next, positive samples were amplified with other genes of a multilocus sequence typing (MLST) system, that is: aspartyl/glutamyl-tRNA(Gln) amidotransferase, subunit B (gatB); cytochrome c oxidase, subunit I (coxA); conserved hypothetical protein (hcpA); and fructose-bisphosphate aldolase (fbpA). In case of multiple infection by different *Wolbachia* supergroups, specific primers were used according to MLST protocols (https://pubmlst.org/*Wolbachia*/info/amp_seq_double.shtml). Infection of *Rickettsia* was screened using a set of primers amplifying the 16S rRNA gene with PCR conditions following (GOTTLIEB *et al*. 2006). Primers for *Spiroplasma* and *Cardinium* 16S rRNA gene were firstly used from FUKATSU *et al.* (2001) and ZCHORI-FEIN & PERLMAN (2004). However, these primers were found to amplify not only targeted bacterial taxa, but also some others (e.g. primers for *Spiroplasma* resulted in sequences identified as *Streptococcus*) or the obtained sequences were of very poor quality, that is chromatograms from these samples contained multiple double peaks or high background, prevents their unambiguous use in further analyses (this concern mainly trials for *Cardinium*). Consequently, new primer sets were designed based on 16S sequences downloaded from GenBank separately for *Spiroplasma* and *Cardinium* (Suppl. Table 2) and used under PCR conditions as follows: 4 min at 95ºC, then 35 cycles of 35 sec at 95ºC, 1 min at 55ºC (both for Spir and Car primers) and 2 min at 72ºC, followed by 10 min at 72ºC.

After DNA purification (Exo-BAP Kit; EURx, Poland), the PCR fragments were sequenced using a BigDye Terminator v.3.1. Cycle Sequencing Kit (Applied Biosystems) and resolved on an ABI 3100 Automated Capillary DNA Sequencer. All newly generated sequences (both from beetles and bacteria) were submitted to GenBank (see Suppl. Table 1) for accession numbers.

Results

Wolbachia infection

Among the 297 examined beetle species, 81 included *Wolbachia*, which gives an infection rate of 27.3% (Table 1). Infection rates in Polyphaga were greater (29%) than in Adephaga (19%). Regarding infraorders, the greatest infection rate was found in Staphyliniformia (54%), followed by Cucujiformia (31%), Scarabaeiformia (23%), Carabiformia (19%), and Elateriformia (5%). No infection was found in Bostrichiformia, but only three species from this infraorder were examined. Infection rates in particular superfamilies were highest in Curculionoidea (59%) and Staphylinoidea (54%), followed by Cucujoidea (25%), Tenebrionoidea (23%), Scarabaeoidea (23%), Chrysomeloidea (20%), Caraboidea (19%), and Buprestoidea (17%) (Fig. 1). No infection was found in examined members of Bostrichoidea, Cleroidea, Dascilloidea, and Elateroidea (all these were examined either in a single species or a few species). At the family level, if considering only those families with more than five examined species, the most infected were: Curculionidae (68%), Staphylinidae (63%), Erotylidae (50%), Apionidae (47%), and Tenebrionidae (43%). Lower infection rates were found in Chrysomelidae (26%), Carabidae (19%), Coccinellidae (19%), and Buprestidae (17%); and no infection was found in, e.g. Cantharidae and Mycetophagidae.

Rickettsia infection

23 species of beetles were infected by *Rickettsia*, which gives an infection rate of 7.7% (Table 1). Infection occurred only in some specimens from every infected species. Almost all infected species (22 of 23) were Polyphaga; only one Adephaga species (*Bembidion articulatum*) was infected (Fig. 1). Regarding infraorders the greatest infection rate was in Elateriformia (20%) followed by Cucujiformia (13%) and Carabiformia (2%). Cleroidea was superfamily with greatest infection rate (40%) followed by Buprestoidea (17%), Curculionoidea (16%), Chrysomeloidea (13%), Cucujoidea (4%) and Caraboidea (2%). (Fig. 1). At the family level Cleroidea dominated with infection rate of 100%, followed by Curculionidae (23%), Buprestidae (17%), Cerambycidae (13%), Chrysomelidae (13%), Apionidae (7%) Coccinellidae (6%) and Carabidae (2%)

Spiroplasma infection

Among examined species nine (3%) were infected by *Spiroplasma* (Table 1). All infected were Polyphaga (Fig. 1): 20% of Elateriformia, 15% of Staphyliniformia, 3% of Scarabaeiformia and 3% of Cucujiformia. Among superfamilies the most infected were Staphylinoidea (15%) followed by Elateroidea (7%), Cucujoidea (4%), Curculionoidea (4%) and Chrysomeloidea (3%) (Fig. 1). At family level the highest infection rate was among Staphylinidae (25%), followed by Cantharidae (13%), Curculionidae (6%), Coccinellidae (6%), Chrysomelidae (5%) and Scarabaeidae (4%).

Cardinium infection

Only 2% (six species from 297) were infected with *Cardinium* (Table 1), all of which belonged to Polyphaga and Cucujiformia (3% infected) (Fig. 1). At superfamily level 8% of Curculionidea and 3% of Chrysomelidea were infected. 33% of family Rhynchitidae, 8% of Curculionidae and 3% of Chrysomelidae carried *Cardinium*.

Co-infection

Eight specimens of the same species were co-infected both by *Rickettsia* and *Wolbachia*: *Aphthona venustula* and *Luperus luperus* (leaf bee-

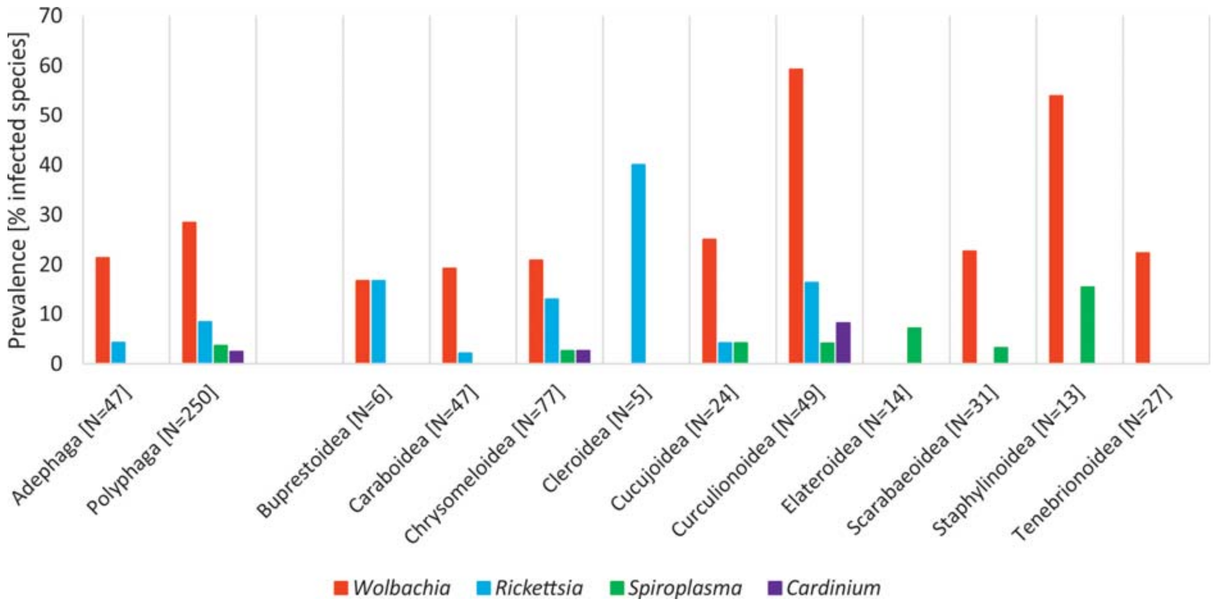

Fig. 1. Prevalence of endosymbiotic “male-killing” bacteria (*Wolbachia*, *Rickettsia*, *Spiroplasma* and *Cardinium*) in 297 species of beetles, illustrated at the level of suborders and superfamilies.

tles), *Mecinus pascuorum*, *Phyllobius glaucus*, *Polydrusus inustus*, *Rhinusa tetra*, *Sitona suturalisa* and *Pseudoprotapion ergenense* (weevils) (Table 1). Four specimens from *Tanymecus palliatus* and *Polydrusus inustus* (weevils), *Psyllobora vigintiduopunctata* (leaf beetle) and *Paederus limnophilus* (rove beetle) were co-infected by *Wolbachia* and *Spiroplasma* (Table 1). Co-infection by *Wolbachia* and *Cardinium* occurred in three species: *Cassida viridis* (leaf beetle), *Sibinia pellucens* (weevil) and *Tanymecus palliatus* (weevil) (Table 1). Finally, we found genes of three bacteria (*Wolbachia*, *Rickettsia* and *Spiroplasma*) in only two weevils: *Polydrusus inustus* and *Tanymecus palliatus*, suggesting that these species could be infected simultaneously by various endosymbionts (Table 1).

Discussion

This is the first study which simultaneously examines infection by all major endosymbiotic “male-killing” bacteria (*Wolbachia, Rickettsia, Spiroplasma* and *Cardinium*) across numerous taxa of coleopterans.

In nearly 300 beetle species, *Wolbachia* infection rate was estimated at approximately 27%, which falls within most calculations for different groups of insects (most estimates suggested between 20% and 70% infection rate (HILGENBOECKER *et al.* 2008; KIKUCHI & FUKATSU 2003; MIURA & TAGAMI 2004) and corresponds well with the average estimate for insects (24% according to ZCHORI-FEIN & PERLMAN 2004 or 23% according to DURON *et al.* 2008). It is also lower than an estimate based on a systematic review across literature about *Wolbachia* infection in beetles by KAJTOCH & KOTÁSKOVÁ (2018), suggesting an infection rate of approximately 38%. However, this estimate was based on numerous previous studies, which usually depended on non-random sampling, i.e. these studies were focused on particular taxonomic group of beetles, which were usually selected on the basis of previous knowledge about *Wolbachia* occurrence. A revision made by KAJTOCH & KOTÁSKOVÁ (2018) indicated that the highest *Wolbachia* infection rates in beetle species were found for the Curculionidae (81 species), Chrysomelidae (49 species), Hydraenidae (14 species), Buprestidae (13 species), Coccinellidae (12 species) and Dytiscidae (8 species). In all other families only 1-3 species were reported to harbor *Wolbachia*. Especially weevils seemed to be a group prone to *Wolbachia* infection according to the literature and the present study. The high prevalence of *Wolbachia* within Curculionidae had been suggested by previous studies, e.g. approx. 40%, according to LACHOWSKA *et al.* (2010) and 34.8% of Scolytinae according to KAWASAKI *et al.* (2016). Wide screening in this study showed even higher values of 68% for Curculionidae and 47% for Apionidae species.

Rickettsia infection was previously found only in single species of Micromalthidae, Staphylinidae, Buprestidae, Coccinellidae and Curculionidae (BILI *et al.* 2016; LAWSON *et al.* 2001;

PEROTTI *et al.* 2016; TOJU & FUKATSU 2011; WEINERT *et al.* 2007; WERREN *et al.* 1994; WHITE *et al.* 2015). In this study the occurrence of this bacteria was extended to five other families: Apionidae, Carabidae, Chrysomelidae, Cerambycidae and Dasytidae. Unfortunately, only a few previous studies examined prevalence of *Rickettsia* in beetles: buprestid beetle (*Brachys tessellatus*) (LAWSON *et al.* 2001) and ladybird beetle (*Adalia bipunctata*) (VON DER SCHULENBURG *et al.* 2001), therefore the estimate of nearly 8% infected species drastically expands knowledge about infection status of *Rickettsia* among beetles. Nearly 8% of infected beetles by *Rickettsia* is much higher than nearly 1% of infected insects found by DURON *et al.* (2008). The value is probably highly underestimated as only single species were screened for *Rickettsia* infection: *Neochrysocharis formosa* (Hymenoptera) (HAGIMORI *et al.* 2006) and *Acyrthosiphon pisum* (Hemiptera) (CHEN *et al.* 1996).

Previous studies (BILI *et al.* 2016; HURST *et al.* 1999a,b; MAJERUS *et al.* 2000; TINSLEY & MAJERUS 2006; TOJU & FUKATSU 2011; WEINERT *et al.* 2007) showed that *Spiroplasma* infects only some species of Staphylinidae, Coccinellidae and Curculionidae. Again, this study substantially extended the number of infected beetles as hosts of *Spiroplasma* were found in Cantharidae, Chrysomelidae and Scarabaeidae. Infection rate at the level of 3% in beetles could not be compared with any other previous estimate due to a lack of data. *Spiroplasma* was found to infect less than 7% of insect species (DURON *et al.* 2008), so infection in beetles is below this average estimate for insects.

Surprisingly, although there was no previous evidence of *Cardinium* infection among beetles (SCHEBECK *et al.* 2018; ZCHORI-FEIN & PERLMAN 2004), our results for 297 beetle species revealed the presence of this bacteria for the first time in beetle hosts. Six species from three families Chrysomelidae, Curculionidae and Rhynchitidae harbored this bacteria. Data about infection rate of *Cardinium* in insects are very limited and only ZCHORI-FEIN & PERLMAN (2004) and DURON *et al.* (2008) suggested that this bacteria infected 6% or 4% of species, respectively. This is quite consistent with the very low infection rate in beetles (2%) found in this study.

Interestingly, in our study co-infection by different endosymbionts occurred rarely and was limited mostly to two bacteria taxa within one host. The only exception was *Polydrusus inustus* and *Tanymecus palliatus*, which were host to three endosymbionts: *Wolbachia*, *Rickettsia* and *Spiroplasma*. This may indicate that there is some balance in the number of these bacteria, probably caused by competition within host cells (GOTO *et al.* 2006). Moreover, specimens from different populations of two species, *Paederus caligatus* and *Paederus limnophilus*, were infected by either *Wolbachia* or *Spiroplasma*, but never by both of these bacteria. On the other hand our results partly correspondence with the study of TOJU & FUKATSU (2011), which indicated that pairs of some endosymbionts (e.g. *Wolbachia–Rickettsia* and *Rickettsia–Spiroplasma*) are more frequent than others. In fact we found eight species that were co-infected by *Wolbachia–Rickettsia,* four by *Wolbachia– Spiroplasma*, but we did not found co-infection by *Rickettsia–Spiroplasma*. There were also only three beetle species which harbored *Cardinium* and any other "male-killing" bacteria (namely *Wolbachia*). However, *Cardinium* as well as *Spiroplasma* were found in a low number of beetle taxa (2% and 3%, respectively), therefore the rare co-infection of these bacteria with other endosymbionts could be simply caused by missing such rare cases.

Beetles are still an insufficiently examined group in terms of interactions with endosymbiotic bacteria, particularly with those described as "male-killers". Such complex group as beetles (expressed by complex phylogenetic relationships and by extensive ecological connections with each other and numerous other species) makes it a perfect group for studying relationships between endosymbionts and their hosts. This should be investigated in the near future, thanks to the development of new molecular tools, such as Next Generation Sequencing, and extending databases of DNA markers useful for identification of taxa and intraspecific diversity. Progress in knowledge about the relationships of intracellular endosymbionts with their hosts should be focused on identification of bacteria diversity at various levels of organization (e.g. by identification and analyses of co-occurrence of distinct strains) and on examination of effects caused by these bacteria (alone or in conjunction) on infected species, populations and individuals.

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Author Contributions

Research concept and design: M.K., D.K., Ł.K.; Collection and/or assembly of data: M.K., D.K., J.M.G., Ś.R., M.A.M., M.H., Ł.K.; Data analysis and interpretation: M.K., Ś.R., M.A.M., Ł.K.; Writing the article: M.K., D.K., J.M.G., Ś.R., M.A.M., M.H., Ł.K.; Critical revision of the article: D.K., J.M.G., Ś.R., M.A.M., M.H., Ł.K.; Final approval of article: M.K., M.A.M., Ł.K.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary Material to this article can be found online at:
http://www.isez.pan.krakow.pl/en/folia-biologica.html

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