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Current state of knowledge on *Wolbachia* infection among Coleoptera: a systematic review

Lukasz Kajtoch Corresp., [1] , **Nela Kotásková** [2]

[1] Institute of Systematics and Evolution of Animals Polish Academy of Sciences, Krakow, Poland

[2] Faculty of Science, University of Ostrava, Ostrava, Czech Republic

Corresponding Author: Lukasz Kajtoch
Email address: kajtoch@isez.pan.krakow.pl

Background. Despite great progress in studies on *Wolbachia* infection in insects, the knowledge about its relations with beetle species, populations and individuals, and the effects of bacteria on these hosts is still unsatisfactory. In this review we summarize the current state of knowledge about *Wolbachia* occurrence and interactions with Coleopteran hosts. **Methods.** An intensive search of the available literature resulted in the selection of 57 publications that describe the relevant details about *Wolbachia* presence among beetles. These publications were then examined with respect to the distribution and taxonomy of infected hosts and diversity of *Wolbachia* found in beetles. Sequences of *Wolbachia* genes (*16S rDNA, wsp* and *ftsZ*) were used for the phylogenetic analyses. **Results.** The collected publications revealed that *Wolbachia* has been confirmed in 152 beetle species and that the estimated average prevalence of this bacteria across beetle species is 36% and varies greatly across families and genera (0-88% infected members) and is much lower (c. 13%) in geographic studies. The majority of the examined and infected beetles were from Europe and East Asia. The most intensively studied have been two groups of herbivorous beetles: Curculionidae and Chrysomelidae, followed by Hydraenidae and Buprestidae. Coleoptera harbor *Wolbachia* belonging to three supergroups: F found in only 3 species, and A and B found in similar numbers of beetles (including some doubly infected); however the latter two were most prevalent in different families. 65% of species with precise data were found to be totally infected. Single infections were found in 69% of species and others were doubly- or multiply-infected. *Wolbachia* caused numerous effects on its beetle hosts, including selective sweep with host mtDNA (found in 4% of species), cytoplasmic incompatibility (detected in c. 7% of beetles) and other effects related to reproduction or development (like male-killing, parthenogenesis reinforcement, possible haplodiploidy induction, and egg development). Phylogenetic reconstructions for *Wolbachia* genes rejected cospeciation between these bacteria and Coleoptera, with minor exceptions found in some closely related Hydraenidae

and Chrysomelidae. In contrast, horizontal transmission of bacteria has been suspected or proven in numerous cases (e.g. among beetles sharing habitats and/or host plants).

Discussion. The present knowledge about *Wolbachia* infection across beetle species and populations is very uneven. Even the basic data about infection status in species and frequency of infected species across genera and families is very superficial, as only c. 0.12% of all beetle species have been tested and/or examined so far. Future studies on *Wolbachia* in Coleoptera using next-generation sequencing technologies will be important for uncovering *Wolbachia* diversity and its relations with host evolution and ecology, as well as with other, co-occurring endosymbiotic bacteria.

Current state of knowledge on *Wolbachia* infection among Coleoptera: a systematic review

Łukasz Kajtoch[1], Nela Kotásková[2]

[1]Institute of Systematics and Evolution of Animals Polish Academy of Sciences, Sławkowska 17, 31-016, Krakow, Poland

[2]Faculty of Science, University of Ostrava,Dvořákova 7, 70103 Ostrava, Czech Republic

corresponding author: Łukasz Kajtoch, lukasz.kajtoch@gmail.com

Abstract

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Key words: α-proteobacteria; beetles; evolution; ecology; endosymbiont; intracellular; interactions

Short title *Wolbachia* among Coleoptera: a review

Introduction

The relations between the intracellular α-proteobacterium *Wolbachia pipientis* Hertig 1936 (hereafter *Wolbachia*) and its hosts from various groups of arthropods and nematodes have been the object of much research and numerous publications (O'Neill et al. 1992; Werren et al. 1995a). The majority of these studies have focused on verifying endosymbiotic bacteria occurrence and diversity in various hosts at different levels: i) among selected species sharing a geographic area (e.g. O'Neill et al. 1992; Werren et al. 1995a, 2000), ii) among species

inhabiting the same environment or that are ecologically-associated (e.g. Stahlhut et al. 2010), iii) among species from particular taxonomic groups (e.g. Czarnetzki et al. 2004, Lachowska et al. 2010, Sontowski et al. 2015), and iv) within populations of selected taxa (e.g. Stenberg et al. 2004, Mazur et al. 2016). Another branch of research on the relations between *Wolbachia* and its hosts has focused on host species phylogenetics or population genetics, which is in some cases related to population differentiation and speciation (e.g. Kubisz et al. 2012; Montagna et al. 2014). In this research *Wolbachia* is sometimes treated as an additional "marker" – a source of genetic data about the eco-evolutionary relations of its hosts. A third type of *Wolbachia* studies has concerned the direct or indirect effects of the infection on host fitness, development or survival at the individual and population levels (e.g. O'Neill 2007; Weeks 2002). Moreover, in a separate branch of research (or in conjunction with the abovementioned types of studies), *Wolbachia* is often examined directly, mainly with respect to strain diversity, distribution and relations with other strains or different co-existing bacteria (Baldo et al. 2007). All these branches of research have substantially extended the knowledge about the relations between the most widespread intracellular endosymbiont – *Wolbachia* and its various hosts. Moreover, these studies have been expanded to encompass other bacteria with similar biologies and effects on hosts (like *Cardinium, Spiroplasma, Rickettsia*) (Duron et al. 2008; Zchori-Fein & Perlman 2004; Goto et al. 2006); however, a great majority of studies are still conducted on *Wolbachia* (Zug et al. 2012). Recently, the various *Wolbachia* supergroups have been proposed to belong to several "*Candidatus* Wolbachia" species (Ramírez-Puebla et al. 2015); however, this approach has been criticized (Lindsey et al. 2016). Due to the uncertain species status of the "*Candidatus* Wolbachia" and because all previous studies considered these presumed different species as distant supergroups, in this review we have followed the previous *Wolbachia* taxonomy.

There are several reviews summarizing the state of knowledge on *Wolbachia* infection among various taxonomic groups of nematodes and arthropods. Over the last years, such reviews have been prepared for the following groups: filarial nematodes (Filarioidea) (Taylor & Hoerauf 1999; Casiraghi et al. 2001), crustaceans (Crustacea) (Cordaux et al. 2001), spiders (Araneae) (Goodacre et al. 2006, Yun et al. 2010),springtails (Collembola) (Czarnetzki et al. 2004), Heteropteran Bugs (Heteroptera) (Kikuchi et al. 2003), wasps (Hymenoptera: Apocrita) (Schoemaker et al. 2002) and butterflies (Lepidoptera) (Tagami et al. 2004). Surprisingly, there is no such review for beetles (Coleoptera), which are the most species rich and diversified group

of organisms on Earth, which are known from most habitats, and whose members belong to all major trophic guilds of animals. Some groups of beetles have been examined with respect to *Wolbachia* infection, but usually only with a limited coverage of species (e.g. weevils, Curculionidae, Lachowska et al. 2010; leaf beetles; Chrysomelidae, Clark et al. 2001, Jäckel et al. 2013; jewel beetles; Buprestidae, Sontowski et al. 2015 and minute moss beetles, Hydraenidae, Sontowski et al. 2015).

In summary, *Wolbachia* has been detected in 10-70% of examined hosts (Jeyaprakash & Hoy 2000; Hilgenboecker et al. 2008), depending on the geographical, ecological or taxonomical association of the selected species. Moreover, more detailed studies, at the population level, have shown that infection is not as straightforward as was assumed in the early stages of *Wolbachia* research. More and more species have been found to be only partially infected, e.g. in only some parts of their ranges or infection was associated with only some phylogenetic lineages (usually correlated with the distribution of mitochondrial lineages) (Clark et al. 2001; Roehrdanz et al. 2006). Furthermore, examples of multiply infected species and individuals have been reported, which has important consequences for the understanding of some of the effects of *Wolbachia* infection (Malloch et al.. 2000; Gurfield 2016). *Wolbachia* is known to have numerous effects on its hosts, among which the most interesting and important are those that disturb host reproduction, such as cytoplasmic incompatibility, thelytokous parthenogenesis, feminization of genetic males, male-killing, increased mating success of infected males via sperm competition and the host's complete dependence on bacteria for egg production (for reviews see Werren 1997; Werren & O'Neill 1997 and Stouthamer et al. 1999). Some of these effects are responsible for diversification of host populations and consequently for 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). This could be another major factor, additional to those already known, responsible for radiation of insects and particularly beetles.

In this review we have summarized the current state of knowledge on the relations between beetles and *Wolbachia* by referring to all the abovementioned groups of research. Moreover, we have highlighted future research directions concerning *Wolbachia* relationships with their diverse Coleopteran hosts.

Survey Methodology

We searched the scientific literature with Google Scholar database, using the following combination of keywords linked by AND (the Boolean search term to stipulate that the record should contain this AND the next term): "*Wolbachia*" AND "Coleoptera" "*Wolbachia*" AND "beetles" and "*Wolbachia*" AND "[names of all beetle families, separately]". Our final literature search for this analysis was conducted on July 5, 2017. Google Scholar has an advantage over other literature databases in that the search term may occur anywhere in the text, instead of just in the title, abstract or keywords. By expert knowledge, we also included other sources like unpublished, accepted articles, dissertations, conference presentations. This produced 113 results. Each result was inspected to determine whether or not it contained information on the subject matter. Articles that had no relevance (e.g., any reports about *Wolbachia*-Coleoptera relations, included only some references to either beetles or bacteria in citations) were excluded. From the remaining articles (n = 70), 13 were excluded as they refer to data already presented in former publications. This collection was biased for literature that had electronic full texts that could be crawled by Google Scholar. The additional documents added through citations and by expert knowledge only partially alleviated this bias. Each document was read critically for the information that it contained on *Wolbachia*-Coleoptera relations, with special reference to answering the study questions listed below. Supplementary Figure 1 shows a flow diagram for the systematic review following Prisma guidelines (Moher et al., 2009).

We examined the collected data on various aspects of *Wolbachia* infection in Coleoptera with respect to the following: the i) characteristics of the publications (to determine the scope and progress of studies on *Wolbachia*) (n=57), ii) geographic distribution of infected beetle species and populations (n=55), iii) sampling design (how many sites and individuals were examined) (n=47), iv) characteristics of the markers (genes) used for genotyping the bacteria (n=55) and their hosts (n=26), v) numbers and frequencies of species found to be infected in particular beetle families and genera (n=48), vi) supergroup prevalence in examined taxonomic groups (n=35), vii) strain distribution and diversity in populations and individuals (n=21), vii) effects of *Wolbachia* on its beetle hosts (n=29). Statistical analyses were done in Statistica 11 (Statsoft).

Finally, we downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and the *Wolbachia* MLST database (https://pubmlst.org/wolbachia/) all available sequences of

Wolbachia genes found in any species of beetle. We restricted further analyses to the most widely used bacteria genes, i.e. *16S rDNA, Wolbachia* surface protein gene *wsp* and cell division protein gene *ftsZ*. Because of the different lengths and spans of available sequences, the long parts of the 3' and 5' ends of each gene were trimmed, which resulted in alignments of length 663 bp for *16S rDNA*, 355 bp for *wsp* and 250 bp for *ftsZ*. The length of the *ftsZ* alignment was particularly short as two different sets of primers have been used for its amplification, and its amplicons only overlapped across a relatively short part of the gene. Phylogenetic trees were only reconstructed for unique gene variants found in particular host taxa. Maximum Likelihood trees were inferred using Maximum Likelihood (ML) implemented in IQ-TREE web serwer http://www.iqtree.org/ (Trifinopoulos et al. 2016) under the following settings Auto selection of substation model, ultrafast bootstrap approximation (UFBoot) (Minh et al. 2013) with 10000 iterations, maximum correlation coefficient = 0.99, single branch test with use of the approximate Likelihood-Ratio Test (SH-aLRT) (Anisimova and Gascuel 2006, Guindon et al. 2010) and other default options.

The nomenclature of host taxa and their systematic positions throughout the paper follow the articles from which the data was derived.

Characterization of *Wolbachia* infection among Coleoptera

Publications

The final list of publications concerning data about *Wolbachia* infection in Coleoptera comprised 57 papers (Supplementary Table 1). The oldest articles with relevant information about *Wolbachia* infection in beetles were published in 1992 (Campbell et al. 1992, O'Neill et al. 1992), and the number of articles since then has increased significantly year by year (Spearman correlation = 0.655; Fig. 1). The majority of these articles (69%) concerned infection in only single beetle species, whereas 15% discussed infection in multiple species belonging to the same genus, 7% − multiple species from the same family, 2% − various species of Coleoptera (only Sontowski et al. 2015) and a further 7% − studies on geographic groups of insects that included some, usually random species of beetles (O'Neill et al. 1992, Werren et al. 1995, 2000).

Most studies were done on Curculionidae (22) and Chrysomelidae (20), following Coccinellidae (6) and Tenebrionidae (6) (Supplementary Table 1). The members of all other

families were investigated in only single studies. Consequently, 2.1 and 2.8 Curculionidae and Chrysomelidae species were respectively examined per article. All species of Hydraenidae and Buprestidae were included in only single articles (Sontowski et al. 2015), whereas limited numbers of species of Coccinellidae and Tenebrionidae were examined in several articles (Majerus et al. 2000, Hurst et al. 1999, Elnagdy et al. 2013, Dudek et al. 2017,Li et al. 2016, Ming et al. 2015, Fialho & Stevens 1996, 1997, 2000). *Wolbachia* infection was only studied more than once in 17 species.

Sampling design

The majority of species investigated with respect to *Wolbachia* infection were from Europe, and a relatively high number of species were from Asia and both Americas, whereas only single articles dealt with African (*Callosobruchus chinensis*, Kondo et al. 2011; *Coccinella undecimpunctata*, Elnagdy et al. 2013; *Hypothenemus hampei*,Vega et al. 2002; *Sitophilus oryzae* and *Sitophilus zaemais*, Heddi et al. 1999) and Australian (*Sitophilus oryzae* and *Sitophilus zaemais*, Heddi et al. 1999) species (Fig. 2). A number of publications describing *Wolbachia* infection in Coleoptera had similar geographic coverages (Fig. 2).

Studies were done on samples collected from an average of 5 sites and concerned on average 54 specimens (Fig. 3). Considering the most widely studied families: Curculionidae and Chrysomelidae, these numbers were on average 7 and 4 sites, respectively, and 77 and 32 individuals, respectively (Fig. 3). The numbers of sites and individuals examined in particular groups were insignificantly different, with the exception of the numbers of examined individuals in Curculionidae and Chrysomelidae (Fig. 3).

Examined genetic markers

The most often used *Wolbachia* gene for studies on Coleoptera was *ftsZ*, followed by *hcpA, wsp* and *16S rDNA* (Fig. 4). Most studies using *hcpA* also used other MLST genes, including *ftsZ.* On the other hand, many species were only investigated with either *16S rDNA* or *wsp* or *ftsZ* alone. Single studies used *groEL* (*Monochamus alternatus*, Aikawa et al. 2009; *Tribolium madens*,Fialho & Stevens 2000) or *ITS* genes (*Tribolium madens*, Fialho & Stevens 2000). So far, only three studies have used next-generation sequencing technology (Illumina) to detect *Wolbachia*; two used *16S rDNA* for metabarcoding of microbiota (*Longitarsus* spp.,

Gurfield 2016; *Harmonia axyridis*, Dudek et al. 2017) and one used shotgun genomic sequencing (*Amara alpine,* Heintzman et al. 2014). For genotyping of hosts, 54.3% of studies utilized fragments of *COI* from mtDNA (usually a barcode fragment of this gene). Fewer studies (25.3%) analyzed *rDNA* (usually *ITS1* and/or *ITS2* spacers),, and only 12.9% and 7.5% of studies used *EF1α* or microsatellites, respectively. In *Wolbachia*-related studies, host genes have been used for several purposes like i) using host DNA as a control for genetic material quality, ii) barcoding for host species identification, iii) phylogenetics, phylogeography and population genetics, iv) estimating co-evolutionary relations between the bacteria and host, and v) detecting some of the effects of *Wolbachia* on its hosts (like linkage disequilibrium, selective sweep, cytoplasmic incompatibility).

Taxonomic coverage

The beetles examined with respect to *Wolbachia* infection belong to 19 families (Gyrinidae, Haliplidae, Noteridae, Dytiscidae, Carabidae, Staphyllinidae, Hydrophilidae, Hydraenidae, Scarabaeidae, Buprestidae, Byturidae, Cleridae, Lampyridae, Coccinellidae, Tenebrionidae, Meloidae, Sylvanidae, Cerambycidae, Chrysomelidae, Curculionidae). In total 152 beetle species were found to harbor *Wolbachia* infection; however the distribution of infected species among families varied markedly. The highest numbers of infected beetle species were found for the Curculionidae (62 species), Chrysomelidae (42 species), Hydraenidae (14 species) and Buprestidae (13 species) (Fig. 5). In all other families only 1-3 species were reported to harbor *Wolbachia* (Supplementary Table 1). However, these numbers are biased by the low number of articles (studies) dealing with members of particular beetle families (see above).

Considering infection across beetle genera, the most richly infected genera were *Altica* (Chrysomelidae, 14 species), *Naupactus* (Curculionidae, 11 species), *Hydraena* (Hydraenidae, 8 species) and *Agrilus* (Buprestidae, 6 species) (Supplementary Table 1). In total, 24 genera were found to have infected members (Fig. 6). The infection in Coleoptera was estimated at 36% of examined species; however, the share of infected species varied greatly between families and genera. At the family level the infection frequency was from 14.3% (Dytiscidae) to 100% (Noteridae) (Sontowski et al. 2015); however when considering only families for which more than 30 species were verified (e.g. Clark et al. 2001, Lachowska-Cierlik et al. 2010, Rodriguer et

al. 2010a, Kondo et al. 2011, Jäckel et al. 2013, Sontowski et al. 2015, Kawasaki et al. 2016), infection was found in up to 63% of species (Hydraenidae) (Table 1). At lower taxonomic levels, *Wolbachia* was found in 25% of Diabroticite (Chrysomelidae; Clark et al. 2001), 14.3-16.7% of Bruchina (Chrysomelidae;Kondo et al. 2011) and 34.8% of Scolytinae (Curculionidae, Kawasaki et al. 2016). Among 47 genera in which *Wolbachia* infection was examined for at least 2 species, 12 genera were completely uninfected, while 6 genera were completely infected (Table 1). If considering only genera with at least 5 verified species, *Wolbachia* was found in 0% (*Acmaeodera*; Buprestidae; Sontowski et al. 2015) to 88% members (*Altica*, Chrysomelidae; Jäckel et al. 2013). There was only a marginally negative and insignificant correlation between the number of examined and number of infected species (R=-0.040). If considering only the most widely examined families: Chrysomelidae and Curculionidae, the difference in infection frequency between these two groups was insignificant (Z=-1.656, P=0.098). Geographic studies on *Wolbachia* prevalence in insects have found much lower frequencies of infection in Coleoptera species: the bacterium was found in only 10.5% of beetles from Panama and 13.5% of beetles from North America (Werren et al. 1995a, 2000).

Wolbachia diversity

Among the various beetle species, *Wolbachia* strains belonged to three supergroups (A, B and F). However, they occurred at very different proportions in different groups of beetles, and these differences were significant (Chi^2=98.78, P=0.000). Overall, the proportion of beetle species found to be infected with *Wolbachia* strains belonging to A or B supergroups was similar, with approx. 18% of all species harboring either supergroup (either as single infections in different species or populations or as multiple infections within individuals) (Fig. 7), whereas F supergroup was found in only 3 beetle species: *Agrilus araxenus* and *Lamprodila mirifica* (both Buprestidae; Sontowski et al. 2015) and *Rhinocyllus conicus* (Curculionidae; Campbell et al. 1992). In the three groups of beetles with the highest numbers of examined and infected species, the distributions of supergroups varied: in Buprestidae, a similar numbers of species were infected by supergroups A and B (all singly infected), with a relatively high share of F infected species (Sontowski et al. 2015). In contrast, in Hydraenida, supergroup A dominated over supergroup B (Sontowski et al. 2015). This was also the case in Chrysomelidae, with some species infected by both strains (Kondo et al. 2011, Jäckel et al. 2013, Kolasa et al. 2017). The

most varied infections were observed in Curculionidae, with supergroup B dominating, a presence of taxa infected by both A and B supergroups, and a single species infected by F supergroup (Lachowska-Cierlik et al. 2010,Rodriguer et al. 2010a,Kawasaki et al. 2016) (Fig. 7). Considering the frequency of infected specimens in the examined beetle species within the available data (N=75), 49 species were reported to be totally infected (all individuals possessed *Wolbachia*), whereas 26 species had this bacterium in only some individuals (Fig. 8). The same calculated for Chrysomelidae resulted in 12 and 9 species, respectively, and for Curculionidae in 33 and 11 species, respectively (Fig. 8). These differences between these values (between these groups of species) was significant (Chi^2=131.89, P=0.000). A single *Wolbachia* strain was observed in 39 species (species with available data N = 56), whereas two strains were reported in 8 species (*Byturus tomentosus*,Malloch et al.. 2000; *Altica quercetorum*, Jäckel et al. 2013; *Callosobruchus chinensis,* Okayama et al. 2016; *Chelymorpha alternans,* Keller et al. 2004;

Crioceris quaterdecimpunctata and *Crioceris quinquepunctata*, Kolasa et al. 2017; *Adalia bipunctata*,Majerus et al. 2000; *Polydrusus inustus*,Kajtoch et al. 2012) and multiple infection in a further 9 species (*Callosobruchus chinensis*,Kondo et al. 2002; *Diabrotica barberi,* Roehrdanz & Levine 2007; *Longitarsus* spp., Gurfield 2016;*Conotrachelus nenuphar* Zhang et al. 2010; *Pityogenes chalcographus*, Arthofer et al. 2009; *Xyleborus dispar* and*Xylosandrus germanus*, Kawasaki et al. 2016) (Fig. 8). In Chrysomelidae (N=19) these numbers were 9, 5 and 5, respectively and in Curculionidae (N=34), 29, 1 and 4, respectively (Fig. 8). The numbers of single, double and multiple infected individuals in these groups of beetles differed insignificantly (Chi^2 ANOVA=0.667, P=0.717).

Effects on hosts

Wolbachia affected beetle hosts in several ways. Linkage disequilibrium and/or selective sweep between bacteria and host genomes (usually with host mtDNA) were detected in 6 species (4%): 2 (5%) Chrysomelidae (*Altica lythri,* Jäckel et al. 2013; *Aphthona nigriscutis*, Roehrdanz et al. 2006) and 4 (6%) Curculionidae (*Eusomus ovulum,* Mazur et al. 2016; *Naupactus cervinus,* Rodriguero et al. 2010b, *Polydrusus inustus, Polydrusus pilifer*, Kajtoch et al. 2012). Cytoplasmic incompatibility was detected in 10 (7%) Coleoptera: 4 (10%) Chrysomelidae (*Altica lythri,* Jäckel et al. 2013; *Aphthona nigriscutis*, Roehrdanz et al. 2006*, Chelymorpha alternans,* Keller et al. 2004*, Diabrotica barberi,* Roehrdanz & Levine 2007, *Diabrotica*

297 *virgifera virgifera*, Clark et al. 2001), 3 (5 %) of Curculionidae (*Cossomus sp.,* Zhang et al.
298 2010*; Hypothenemus hampei,*Mariño et al. 2017, *Xylosandrus germanus*, Kawasaki et al. 2016),
299 1 of Scarabaeidae (*Popillia japonica*, Jensen 2011), 1 of Sylvanidae (*Oryzaephilus surinamensis*,
300 Sharaf et al. 2010) and 1 of Tenebrionidae (*Tribolium confusum*, Li et al. 2016, Ming et al.
301 2015). Horizontal transfer of *Wolbachia* was detected or suspected in 28 species of Coleoptera
302 (19%) − 16 (39%) species of Chrysomelidae (several species of *Altica,* Jäckel et al. 2013,
303 *Crioceris quaterdecimpunctata* and *Crioceris quinquepunctata*, Kolasa et al. 2017) and 12 (19%)
304 species of Curculionidae(members of *Euwallacea,Xyleborus, Xylosandrus*, *Xyleborinus*
305 *schaufussi* and *Taphrorychus bicolor* Kawasaki et al. 2016, *Polydrusus* and *Parafoucartia*
306 *squamulata,* Kajtoch et al. 2012). Other effects of *Wolbachia* on beetles included the following:
307 i) transfer of bacteria genes to the autosomes of the host (so far detected only for *Monochamus*
308 *alternatus*, Cerambycidae, Aikawa et al. 2009 and *Callosobruchus chinensis*, Chrysomelidae,
309 Nikoh et al. 2008); ii) coexistence of *Wolbachia* with *Rickettsia* (*Longitarsus,* Chrysomelidae,
310 Gurfield 2016) in the host or with *Rickettsia* and *Spiroplasma* (*Adalia bipunctata,* Majerus et al.
311 2000, *Harmonia axyridis*, Dudek et al. 2017; both Chrysomelidae); iii) induction and
312 reinforcement of parthenogenesis (numerous species of Naupactini, Rodriguer et al. 2010a and
313 *Eusomus ovulum*, Mazur et al. 2016; all Curculionidae); iv) possible induction of haplodiploidy
314 (*Euwallacea interjectus, Euwallacea validus*, Curculionidae, Kawasaki et al. 2016); v) male-
315 killing (*Tribolium madens*, Tenebrionidae, Fialho & Stevens 2000); vi) necessity of infection for
316 egg development (*Otiorhynchus sulcatus*, Curculionidae, Son et al. 2008); vii) populations
317 evolving towards endosymbiont loss and repeated intraspecific horizontal transfer of *Wolbachia*
318 (*Pityogenes chalcographus,* Curculionidae, Arthofer et al. 2009).

319 *Phylogenetic relations*

320 The tree reconstructed for *16S rDNA* included 43 sequences from bacteria found in 36
321 host beetle species. This tree included three major lineages, with separate clusters of *Wolbachia*
322 sequences belonging to A, B and F supergroups (Supplementary Fig. 2). F supergroup was
323 represented by a single sequence from *Rhinocyllus conicus* (Curculionidae) (Supplementary Fig.
324 2). Sequences assigned to supergroup A (based on information available in the articles) were
325 found to be polyphyletic. Some *16S* sequences from *Xylosandrus germanus* (Curculionidae) and
326 *Oreina cacaliae* (Chrysomelidae) clustered as a sister lineage to all other A and B sequences, and

appeared as an intermediate between supergroup F and other supergroups (Supplementary Fig. 2). Overall, the diversity of *16S* sequences assigned to supergroup B was much greater than those assigned to supergroup A (Supplementary Fig. 2).

The tree reconstructed for *ftsZ* included 121 sequences found in 104 host beetle species. The *ftsZ* phylogenetic tree resulted in a topology similar to that of *16S rDNA* − it included groups of sequences belonging to A, B and F supergroups (Supplementary Fig. 3). Supergroup F was represented by *Agrilus araxenus* and *Sphaerobothris aghababiani* (both Buprestidae). Moreover, the supergroup B clade was divided into two clusters, among which one included a small group of sequences found in four beetle hosts: *Chelymorpha alternans* (Chrysomelidae)*, Eurymetopus fallax, Sitophilus oryzae* and *Conotrachelus nenuphar* (all three Curculionidae) (Supplementary Fig. 3). Also in this gene, the genetic variation of sequences belonging to supergroup A was much lower, and only a few sequences were highly diverged (e.g. strains of *Callosobruchus chinensis*, Chrysomelidae; *Tribolium confusum*, Tenebrionidae or *Polydrosus pilosus,* Curculionidae) (Supplementary Fig. 3). There was also one slightly distinct clade that mainly consisted of bacteria sequences found in some Hydraenidae, Curculionidae and Chrysomelidae (Fig. 10).

The *Wsp* tree was built for 83 sequences found in 63 hosts. This network resulted in two clusters representing supergroups A and B; among the available sequences there were no representatives of supergroup F (Supplementary Fig. 4). *Wsp* was found to be more diverse than *16S* and *ftsZ*, as it had multiple distant lineages in both supergroups. Within supergroup B the most distant lineage originated from the only *wsp* sequence found in *Callosobruchus analis* (Chrysomelidae) (Supplementary Fig. 4). In this supergroup, two distinct clades could also be delineated: one consisting of *Wolbachia* sequences found in a variety of beetle hosts and the second mainly consisting of hosts from Curculionidae (*Otiorhynchus singularis, Sitophilus* spp.), Chrysomelidae (*Callosobruchus* spp., *Acromis sparsa*) and Byturidae (*Byturus tomentosus*) (Supplementary Fig. 4). Similarly, in supergroup A several distinct lineages could be delineated, consisting of *Wolbachia* sequences found in e.g. *Ceutorhynchus obstrictus* (Curculionidae), *Diabrotica* spp., *Oreina* spp. and *Aphthona* spp. (all Chrysomelidae) – which are all represented by several strains (Supplementary Fig. 4).

The abovementioned phylogenetic reconstructions of the relations among *Wolbachia* strains identified on the basis of polymorphism of several genes show that there is no strict correlation between host phylogeny and bacterial strain relationships. Even in studies that covered multiple related species (e.g. those belonging to the same genus), evidence for direct inheritance of *Wolbachia* strains from common ancestors is restricted to Hydraenidae (Sontowski et al. 2015) and some species of *Oreina* (Montagna et al. 2014). In the case of *Altica,* the data show that cospeciation was rare and restricted to a few recently diverged species (Jäckel et al. 2013). In contrast, there are numerous examples of phylogenetically related beetle species possessing different *Wolbachia* strains (e.g. Lachowska et al. 2010). It is also often the case among related species that some are infected, whereas others not (*Crioceris*, Kubisz et al. 2012; *Oreina,* Montagna et al. 2014); so any assumption that the bacteria was inherited from a common ancestor would also need to consider multiple losses of infection. The latter phenomenon is probable; however, there is no direct evidence from natural populations, at least in studies on beetles, of *Wolbachia* disappearing over time. Some exemplary studies that found *Wolbachia* present in related species, after detailed examination, rejected the idea that bacteria was inherited from a common ancestor. This was because different host species harbored unrelated stains (e.g. among weevils, Lachowska et al. 2010, Rodriguer et al. 2010a) or in cases where strains were identical or similar, the hosts were not phylogenetically close to each other (e.g. *Crioceris*, Kubisz et al. 2012). Finally, there is evermore proof of horizontal *Wolbachia* transmission via different mechanisms, such as via predators, parasitoids, common habitat or foraging on the same host plants (Huigens et al. 2004,Stahlhut et al. 2010,Caspi-Fluger et al. 2012,Ahmed et al. 2015). Studies on beetles have mainly provided indirect evidence of such transmissions. There are known groups of species that inhabit the same environments and share the same or very similar *Wolbachia* strains, e.g. steppic weevils from East-central Europe (Mazur et al. 2014) and bark beetles in Japane (Kawasaki et al. 2016). Recently, proof has also appeared for the role of host plants in bacteria spread – *Wolbachia* DNA was detected in two species of *Crioceris* leaf beetles and in their host plant – *Asparagus* spp. (Kolasa et al. 2017).

Current gaps and future endeavors

The present knowledge on *Wolbachia* infection across beetle species and populations is very uneven. Even the basic data about infection statuses in species and frequencies of infected

386 species across genera and families is superficial, as there are only c. 150 beetle species known to
387 be infected. This means that if 36% is the average frequency of infection among beetle species,
388 then only c. 420 species have been tested so far. This is merely c. 0.12% of the total number of
389 beetles, which is estimated to be around 360 000 species (Farrell 1998, Bouchard et al. 2009).
390 We know even less at the population level, as the majority of beetle species have only had single
391 individuals tested for *Wolbachia* infection (e.g. Lachowska et al. 2010, Sontowski et al. 2015).
392 These very basic screens have probably underestimated the number of infected species because
393 of false-negative results obtained for species with low or local infection in populations. On the
394 other hand, these preliminary estimates could have overestimated the real number infected
395 beetles, as sampling in these studies was rarely random and most often focused on specific
396 groups, e.g. on genera for which preliminary data suggested the presence of *Wolbachia* infection.
397 Indeed, an intensive search of *Wolbachia* infection across hundreds of beetle species from
398 Europe suggested a lower infection rate – c. 27% to be infected. Also, knowledge about infection
399 at the geographic scale is very uneven, and only Europe and Asia (basically China and Japan)
400 have been relatively well investigated. There is a huge gap in the knowledge for African,
401 Australian and Oceanian beetles, where a high diversity of beetles exists and probably a similar
402 diversity of *Wolbachia* could be expected (e.g. compared to preliminary data available from
403 Central and South America (Werren et al. 1995, Rodriguer et al. 2010a)).

404 Little is known about *Wolbachia* diversity in beetle hosts, as the majority of studies used
405 only single genetic markers, and often different genes were sequenced for different taxa. This
406 precludes complex analysis of *Wolbachia* diversity across all tested beetle hosts. This has
407 changed since 2006, since Baldo et al. (2006) proposed Multilocus Sequence Typing, which is
408 based on the genotyping of five housekeeping genes, usually in conjunction with *wsp*
409 sequencing. But this remains a superficial way for understanding *Wolbachia* diversity as the
410 genome of this bacteria is known to be affected by frequent recombination events (Werren et al.
411 1995, Werren & Windsor 2000). To fully understand *Wolbachia* diversity and relations among
412 strains and supergroups, genome-sequencing is needed. This could be achieved thanks to the
413 development of next-generation sequencing technologies (NGS). Surprisingly, despite fast
414 development of NGS in the last years, very few studies have used this technology for studying
415 *Wolbachia* in beetle populations. For example, two studies sequenced *16S* amplicons generated
416 from microbiota and accidentally detected *Wolbachia* (Gurfield 2016; Dudek et al. 2017). The

only single study that utilized shotgun sequencing was executed for other purposes and also accidentally showed *Wolbachia* genes in examined species (Heintzman et al. 2014). NGS is probably the best prospect for studies on *Wolbachia* infection and diversity, and will help to answer most current riddles and issues.

The big challenge is to understand the impact of infection on beetle biology, physiology and ecology. It is known that *Wolbachia* has several effects on host reproduction, but relatively few studies prove or suggest e.g. cytoplasmic incompatibility, male-killing or other effects on the development of selected beetles (Jäckel et al. 2013, Roehrdanz et al. 2006, Keller et al. 2004, Roehrdanz & Levine 2007,Clark et al. 2001,Zhang et al. 2010; Mariño et al. 2017,Kawasaki et al. 2016,Jensen 2011, Sharaf et al. 2010, Li et al. 2016, Ming et al. 2015). It is very probable that this bacteria has large and frequent effects on beetle reproduction and is consequently partially responsible for beetle radiation, at least in some taxonomic groups, geographic areas or habitats. Also very few studies have shown data on linkage disequilibrium and selective sweep between bacteria and host genomes (Jäckel et al. 2013,Roehrdanz et al. 2006, Mazur et al. 2016, Rodriguero et al. 2010b,Kajtoch et al. 2012). These effects could also have led to the speciation of numerous beetles. Moreover, this phenomenon could have serious implications for beetle barcoding, as selective sweep is known to reduce mitochondrial diversity in its hosts and therefore could decrease the number of identified species (Hurst & Jiggins 2005). On the other hand, cytoplasmic incompatibility can lead to the origin of highly diverged phylogenetic mitochondrial lineages within species, which would increase the number of identified taxa (Smith et al. 2012). Also here, NGS technologies will enable more sophisticated analyses of these genetic relations and their effects (e.g. by the sequencing of transcriptomes for physiological studies or by genotyping-by-sequencing for phylogenetic studies). Genotyping with NGS should also verify whether the recent assumption that different supergroups are indeed "*Candidatus* Wolbachia" species is correct or not (Ramírez-Puebla et al. 2015, Lindsey et al. 2016).

Only very preliminary results suggested *Wolbachia* was not only transmitted vertically, but that it could also have spread horizontally (Jäckel et al. 2013, Kolasa et al. 2012, 2017, Kawasaki et al. 2016, Mazur et al. 2017). In light of the general lack of cospeciation between bacteria and beetles, horizontal transmission must be a highly underestimated phenomenon.

Horizontal transmission of Wolbachia among beetles, cannot be confirmed without considering other coexisting insects that can mediate transmission, such as predators, parasitoids or beetle prey. Moreover, other arthropods that share habitats with beetles, e.g. phoretic ticks (Hartelt et al. 2004) and nematodes (Casiraghi et al. 2001), need to be examined. Finally, host plants are promising objects of studies on *Wolbachia* transmission across beetle populations (Kolasa et al. 2017), as phloem is probably an important mediator of this bacteria's spread across insect populations (DeLay 2012; Li et al. 2016). Concerning transmission – another very poorly investigated topic is the transfer of *Wolbachia* genes into host genomes, as only two such examples have been reported so far (Aikawa et al. 2009, Nikoh et al. 2008). This problem could be important as if such transfers are frequent, simple testing of *Wolbachia* presence in a host based on single or even several gene sequencing could overestimate the number of truly infected species, populations or individuals.

Finally, a very interesting topic for future studies is the examination of the presence of other intracellular and symbiotic bacteria (like *Cardinium, Spiroplasma, Rickettsia*), in Coleoptera and their relations, both with the host and *Wolbachia*. So far, only three studies have found *Wolbachia* with *Rickettsia* and/or *Spiroplasma* together in beetle hosts (Gurfield 2016,Majerus et al. 2000, Dudek et al. 2017). Preliminary results suggest that there is some balance in the number of these bacteria, probably caused by competition within host cells (Goto et al. 2006). A recent summary of the presence of these bacteria in insects showed that *Rickettsia* has been found in single species of Buprestidae and Coccinellidae (Werren et al. 1994, Lawson et al. 2001), *Spiroplasma* in some species of Coccinellidae (Majerus et al. 1998, Hurst et al. 1999, Tinsley & Majerus 2006), and *Cardinium* has not been detected so far in any beetle species (Zchori-Fein et al. 2004). The coexistence of different endosymbiotic bacteria and their effects on hosts should also be investigated with NGS technologies, which are able to detect bacteria in numerous hosts (e.g. individuals) and estimate prevalence of bacteria in various hosts or different tissues. Similar or opposite effects of different endosymbiotic bacteria on beetle species, populations and individuals could be the greatest overlooked phenomenon in the evolution and ecology of Coleoptera.

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Table 1(on next page)

Image of share of *Wolbachia* infected species among families and genera of examined beetles.

Table 1: Share of *Wolbachia* infected species among families and genera of examined beetles. Only taxonomic groups for which at least two species were tested are presented.

1

family	N of examined	% of infected	genus	N of examined	% of infected	genus	N of examined	% of infected
Buprestidae	61	23.0	*Barypeithes*	9	11.0	*Julodis*	2	0.0
Chrysomelidae	81	45.7	*Brachysomus*	4	0.0	*Laccophilus*	2	0.0
Curculionidae	92	51.1	*Buprestis*	3	0.0	*Limnebius*	7	28.6
Dytiscidae	21	14.3	*Byturus*	3	33.0	*Longitarsus*	3	100.0
Gyrinidae	3	33.3	*Callosobruchus*	7	33.0	*Meliboeus*	2	0.0
Haliplidae	2	50.0	*Capnodis*	3	33.3	*Naupactus*	16	69.0
Hydraenidae	27	63.0	*Charidotella*	2	50.0	*Neoglanis*	2	0.0
Hydrophilidae	12	16.7	*Chrysobothris*	3	33.3	*Ochthebius*	12	41.7
Noteridae	2	100.0	*Crioceris*	5	40.0	*Oreina*	5	80.0
genus	N of examined	% of infected	*Diabrotica*	12	25.0	*Otiorhynchus*	4	50.0
Acalymma	2	100.0	*Dorytomus*	3	67.0	*Pantomorus*	3	100.0
Acmaeodera	5	0.0	*Eurymetopus*	2	100.0	*Polydrosus*	4	75.0
Acmaeoderella	4	0.0	*Gyrinus*	3	33.0	*Rhantus*	2	0.0
Agabus	6	16.7	*Haliplus*	3	33.0	*Sciaphobus*	2	50.0
Agrilus	34	17.6	*Helophorus*	3	0.0	*Sitophilus*	3	100.0
Altica	16	88.0	*Hydraena*	24	33.3	*Sphenoptera*	11	9.1
Anthaxia	6	16.7	*Hydroporus*	5	0.0	*Strophosoma*	3	67.0
Aramigus	3	100.0	*Hygrotus*	5	20.0	*Trachypteris*	2	0.0
Atrichonotus	2	50.0	*Ilybius*	2	0.0	*Trachys*	6	16.7

2

3

Figure 1(on next page)

Prisma flow-diagram for literature on Wolbachia-Coleoptera relations included in this study.

Figure 1: Prisma flow-diagram (see Moher et al., 2009) for literature on Wolbachia-Coleoptera relations included in this study.

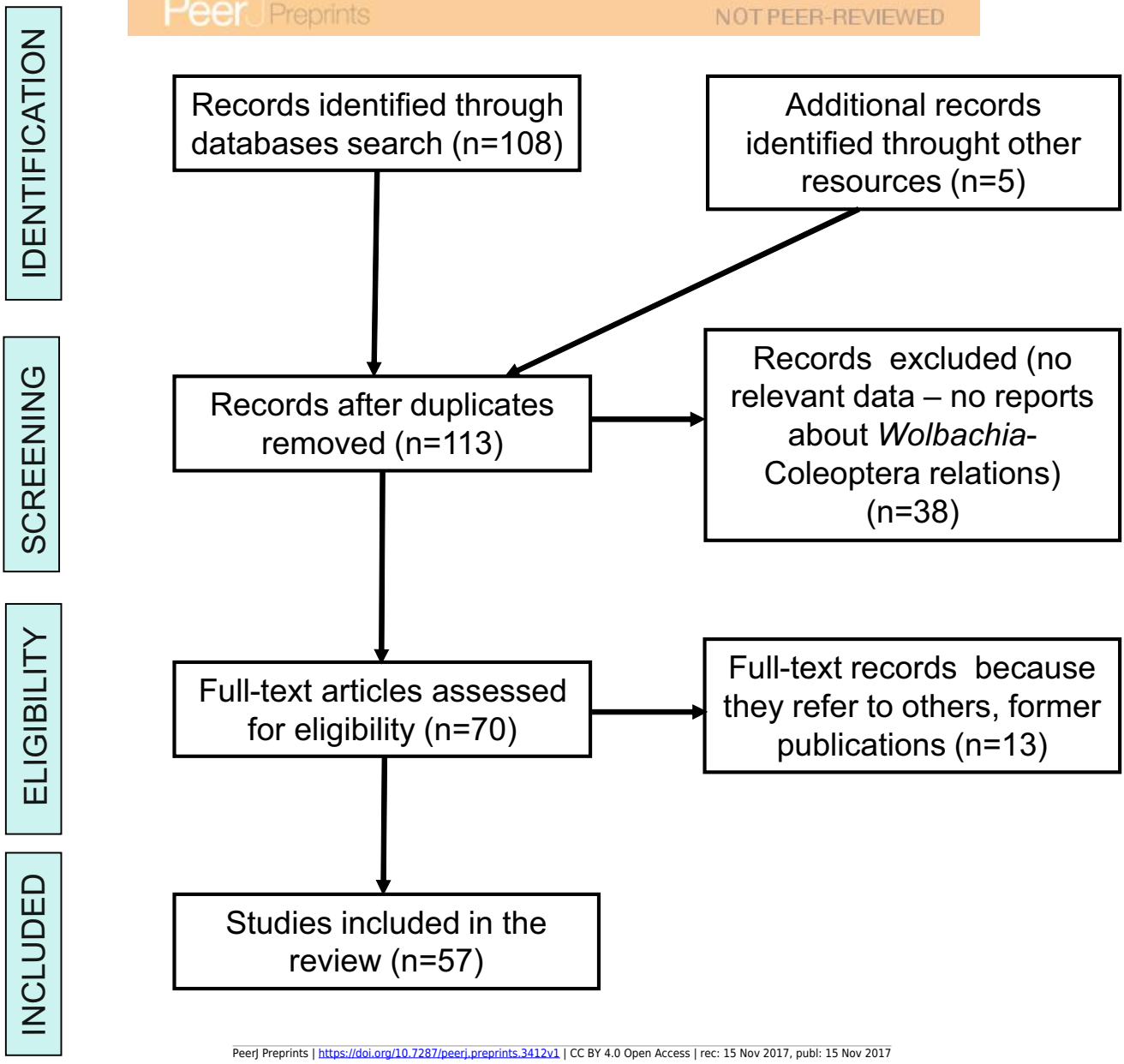

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Figure 2(on next page)

Image of change in the number of publications considering *Wolbachia* infection among Coleoptera.

Figure 2: Change in the number of publications considering *Wolbachia* infection among Coleoptera.

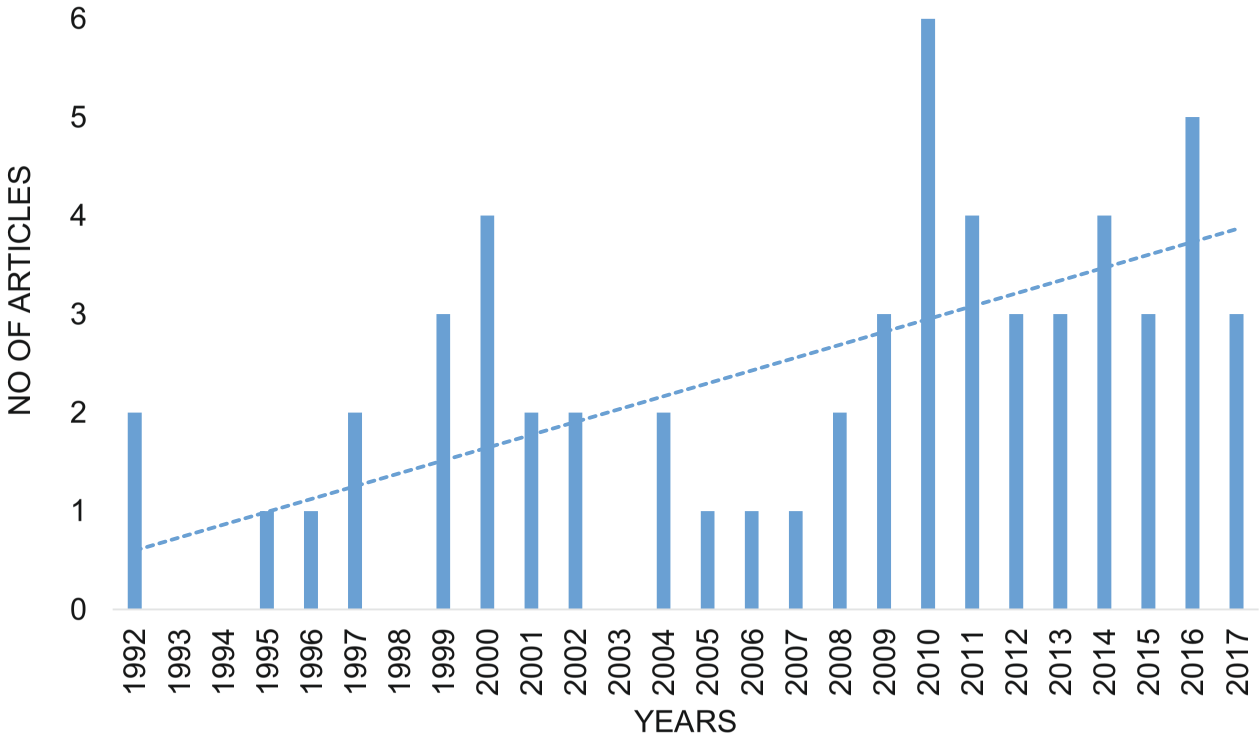

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Figure 3(on next page)

Image of number of publications that described *Wolbachia* infection among Coleoptera and number of infected beetle species.

Figure 3: Number of publications that described *Wolbachia* infection among Coleoptera and number of infected beetle species. Both are shown with respect to the zoogeography of the examined hosts (from which continent the host was collected).)

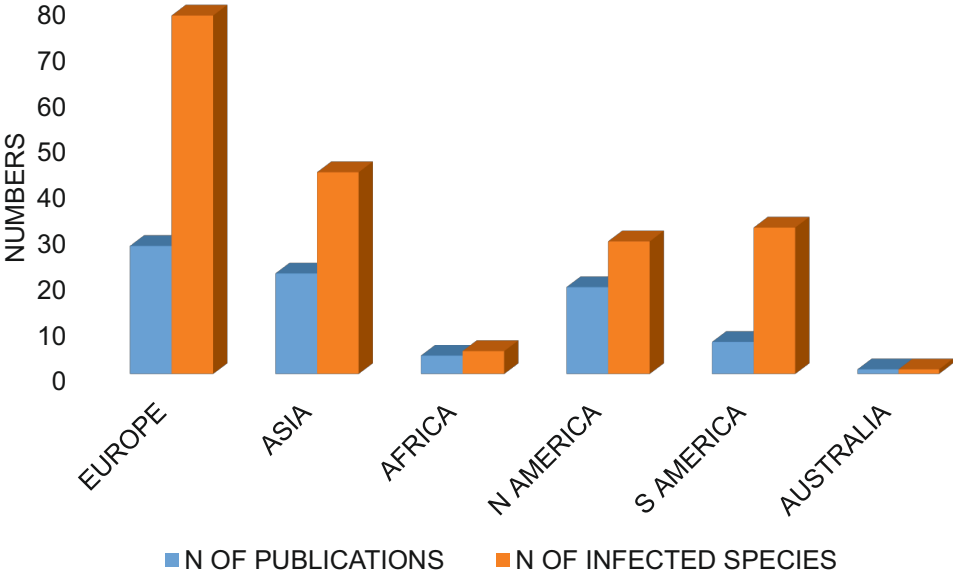

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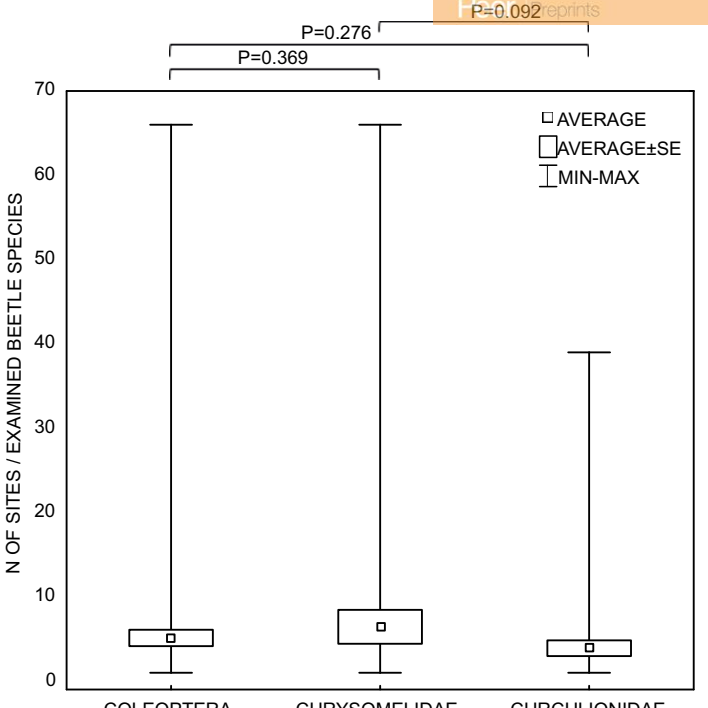
Figure 4(on next page)

Image of numbers of sites and numbers of individuals of beetles examined with respect to *Wolbachia* infection.

Figure 4: Numbers of sites and numbers of individuals of beetles examined with respect to *Wolbachia* infection. P – Man-Whitney test p-values.



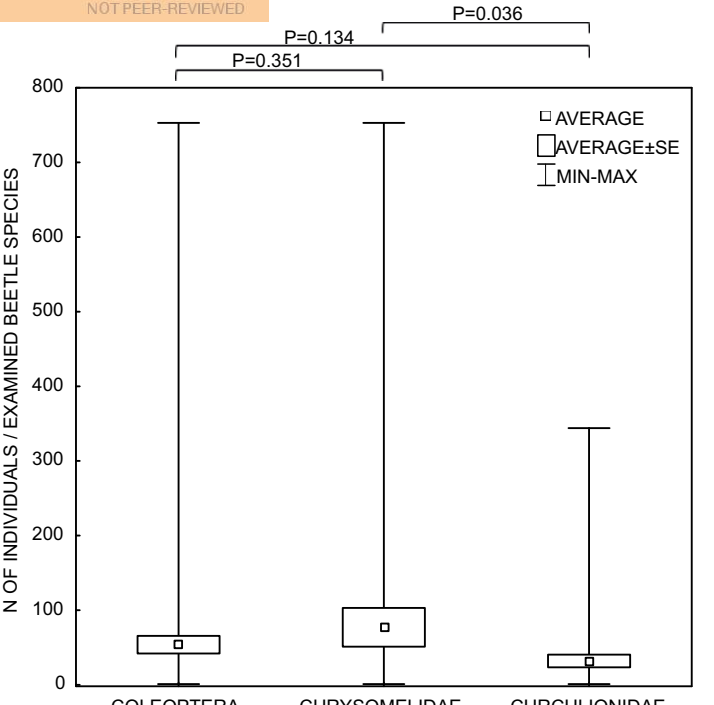


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Figure 5(on next page)

Image of shares of *Wolbachia* genes used in studies on *Wolbachia* infection among Coleoptera.

Figure 5: Shares of *Wolbachia* genes used in studies on *Wolbachia* infection among Coleoptera.

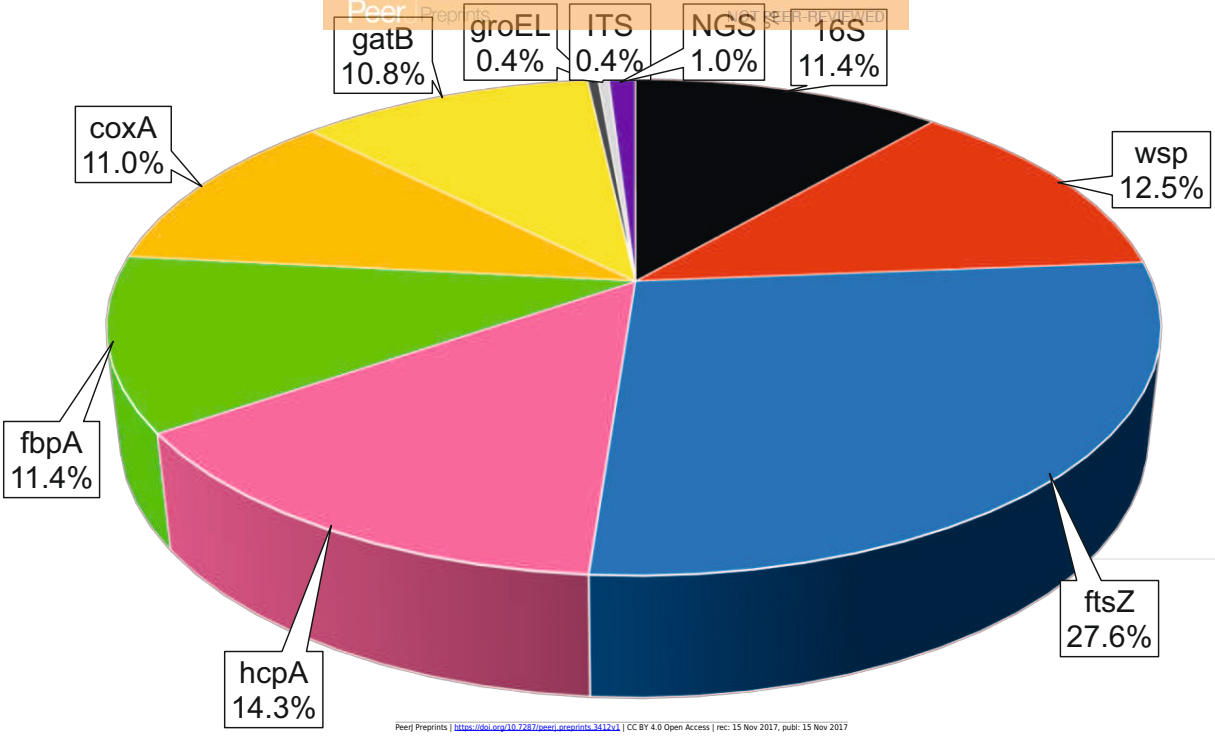

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Figure 6(on next page)

Image of shares of *Wolbachia* infected beetle species across the examined families of Coleoptera.

Figure 6: Shares of *Wolbachia* infected beetle species across the examined families of Coleoptera. The numbers presented after the family names indicate the number of infected species.

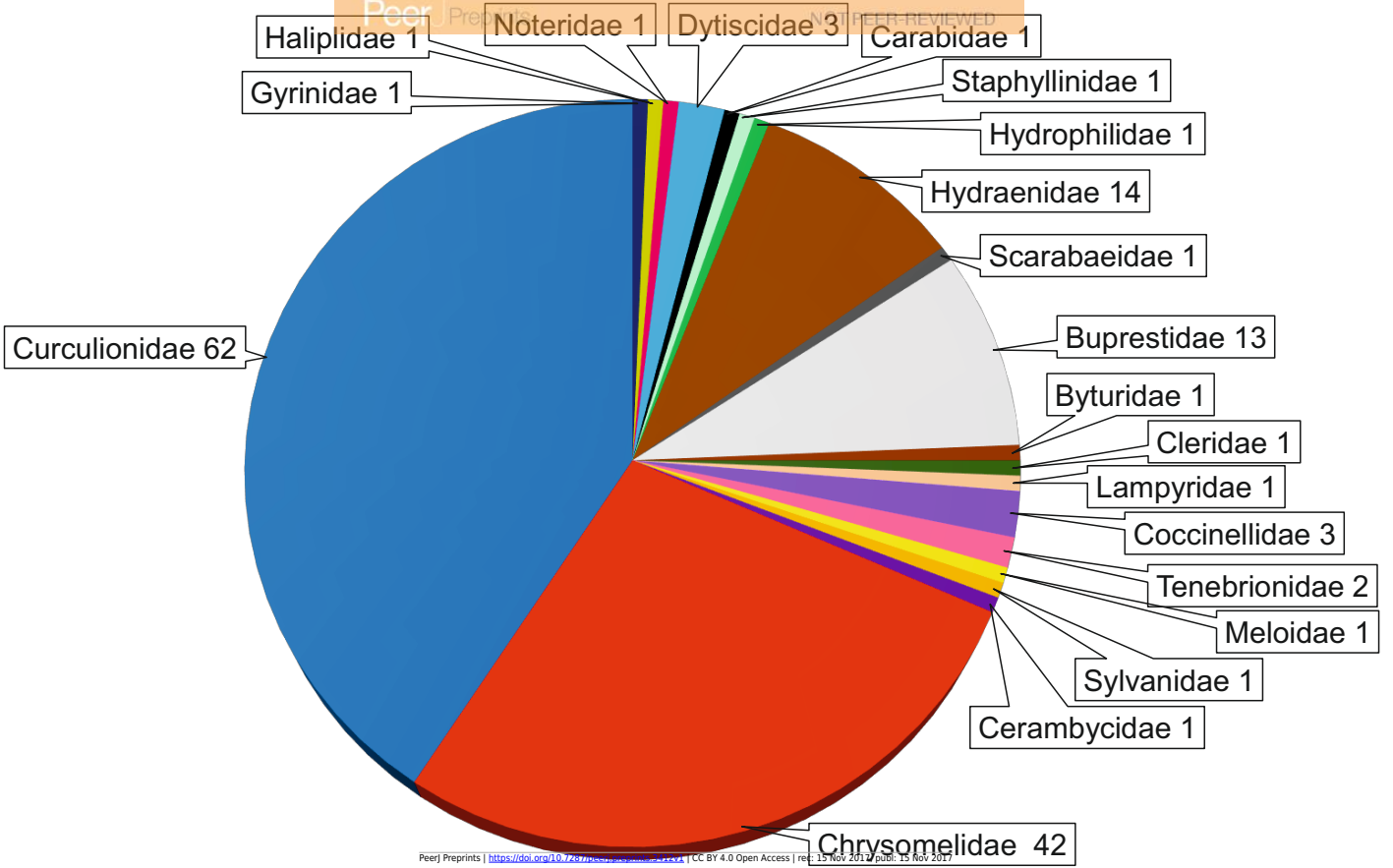

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Figure 7 (on next page)

Image of numbers of *Wolbachia* infected species found in the examined genera of beetles.

Figure 7: Numbers of *Wolbachia* infected species found in the examined genera of beetles.

Only genera with at least two infected species are presented.

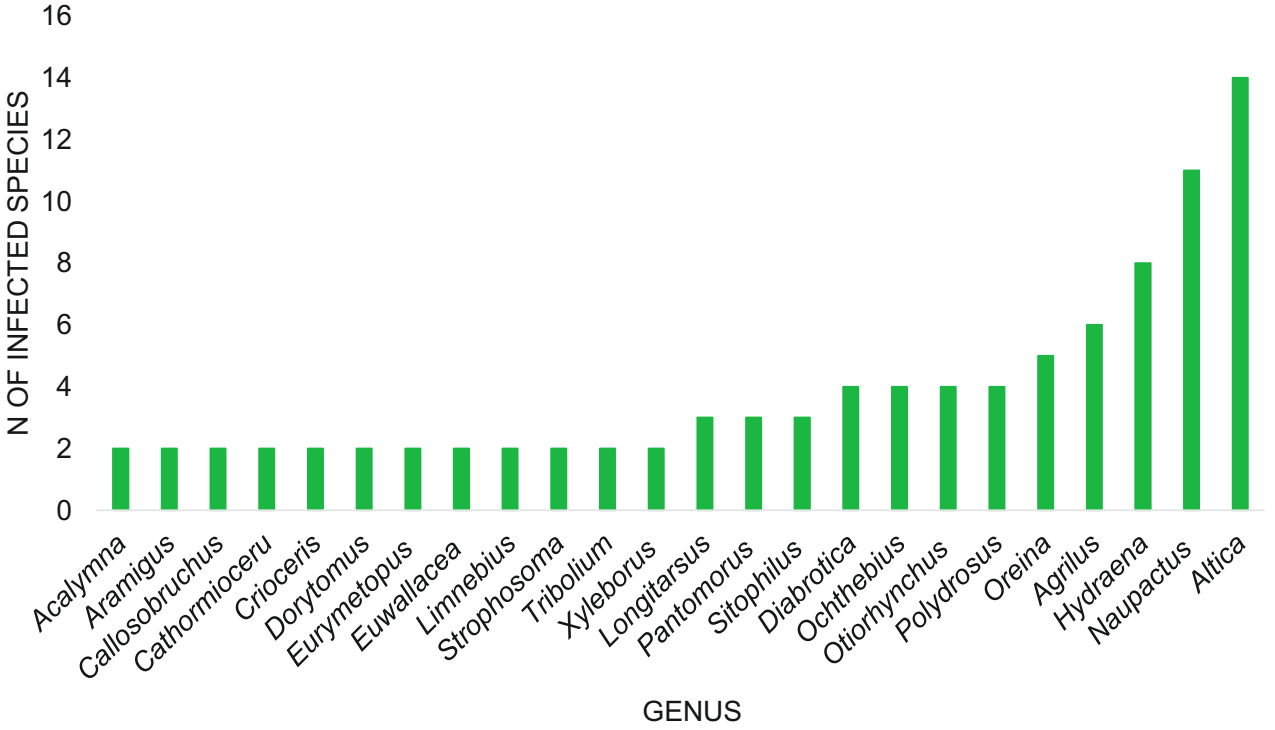

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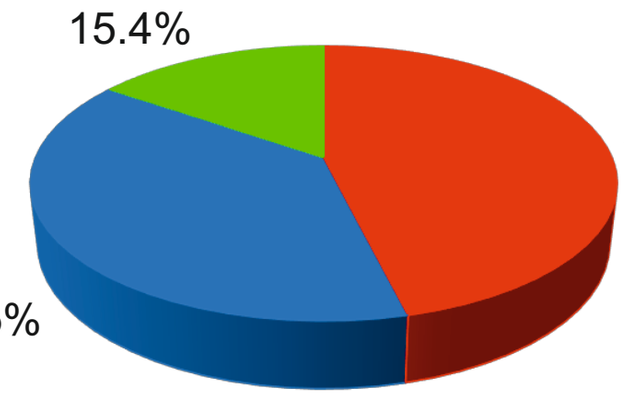
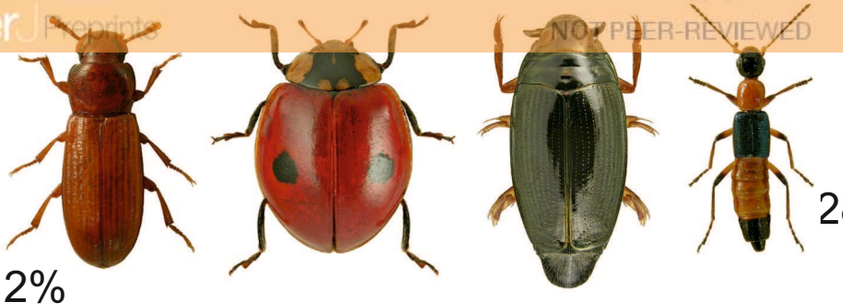
Figure 8(on next page)

Image of shares of beetles infected by *Wolbachia* supergroups (A, B, F).

Figure 8: Shares of beetles infected by *Wolbachia* supergroups (A, B, F). [Beetle photographs are from ICONOGRAPHIA COLEOPTERORUM POLONIAE (© Copyright by Prof. Lech Borowiec]

BUPRESTIDAE





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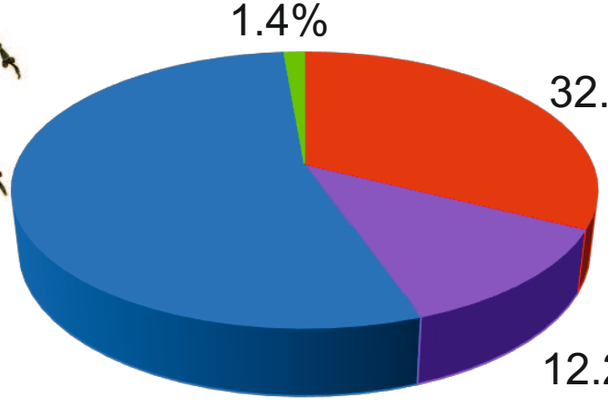
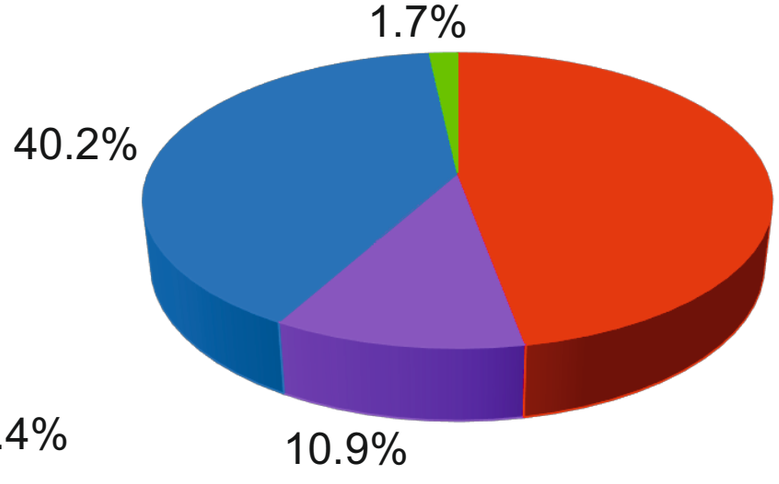


COLEOPTERA



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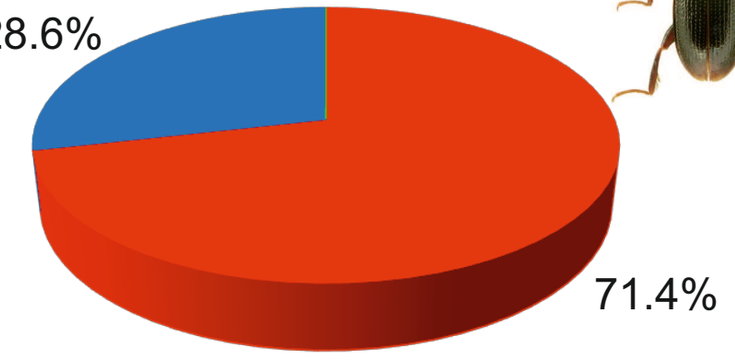

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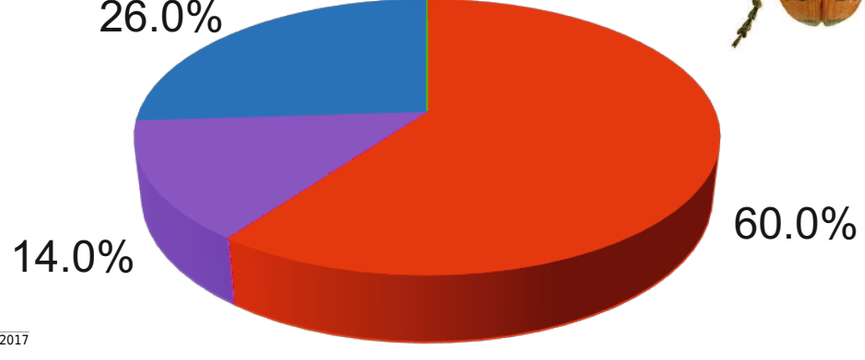

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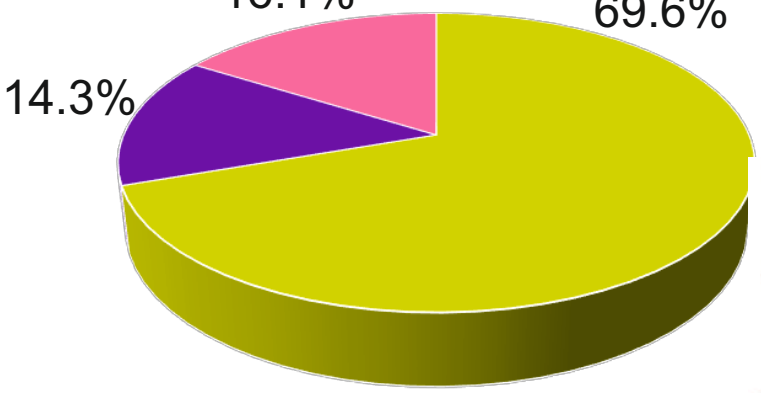
Figure 9(on next page)

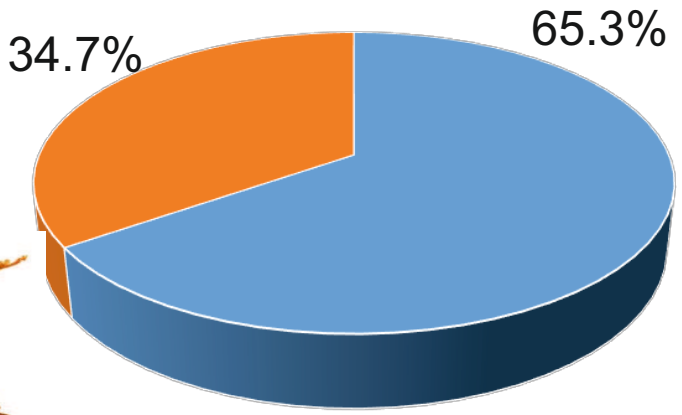
Image of diversity of *Wolbachia* infection in Coleoptera with respect to shares of infected individuals within species and numbers of strains found in beetles.

Figure 9: Diversity of *Wolbachia* infection in Coleoptera with respect to shares of infected individuals within species and numbers of strains found in beetles. [Beetle photographs are from ICONOGRAPHIA COLEOPTERORUM POLONIAE (© Copyright by Prof. Lech Borowiec]

COLEOPTERA



COLEOPTERA



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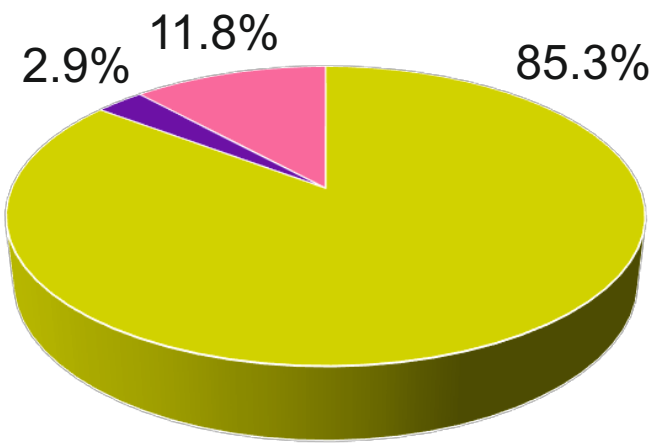

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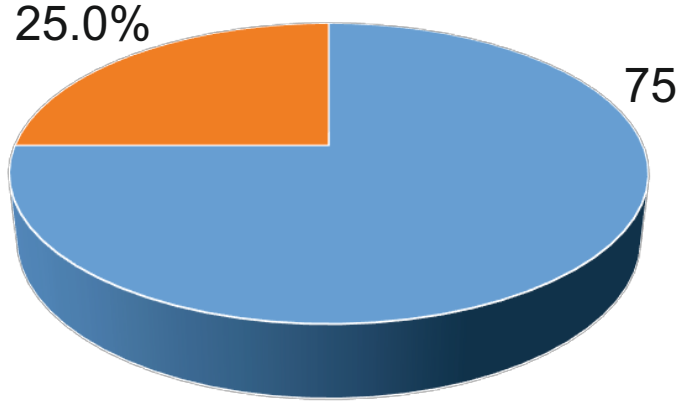

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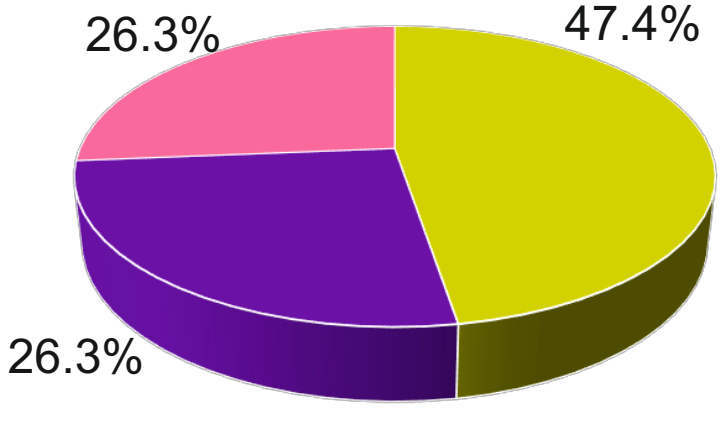

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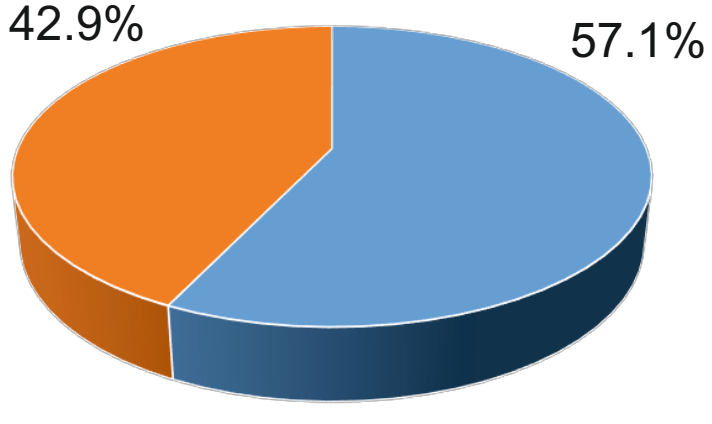

1 STRAIN 2 STRAINS MULTIPLE STRAINS ALL INFECTED SOME INFECTED