1   *Arsenophonus* and *Sodalis* replacements shape evolution

2   of symbiosis in louse flies

3   Eva Šochová[1], Filip Husník[2, 3, †], Eva Nováková[1, 3], Ali Halajian[4], Václav Hypša[1, 3]

4   [1]Department of Parasitology, University of South Bohemia, České Budějovice, Czech

5   Republic

6   [2] Department of Molecular Biology, University of South Bohemia, České Budějovice, Czech

7   Republic

8   [3] Institute of Parasitology, Biology Centre, ASCR, v.v.i., České Budějovice, Czech Republic

9   [4] Department of Biodiversity, University of Limpopo, Sovenga 0727, South Africa

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11   Corresponding author:

12   Eva Šochová[1]

13   Email address: sochova.e@seznam.cz

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[†] Present address: University of British Columbia, Vancouver, British Columbia, Canada

21 **Abstract**

22 Symbiotic interactions between insects and bacteria are ubiquitous and form a continuum from

23 loose facultative symbiosis to greatly intimate and stable obligate symbiosis. In blood-sucking

24 insects living exclusively on vertebrate blood, obligate endosymbionts are essential for hosts

25 and hypothesized to supplement B-vitamins and cofactors missing from their blood diet. The

26 role and distribution of facultative endosymbionts and their evolutionary significance as seeds

27 of obligate symbioses are much less understood. Here, using phylogenetic approaches, we

28 focus on the Hippoboscidae phylogeny as well as the stability and dynamics of obligate

29 symbioses within this bloodsucking group. In particular, we demonstrate a new potentially

30 obligate lineage of *Sodalis* co-evolving with the Olfersini subclade of Hippoboscidae. We also

31 show several likely facultative *Sodalis* lineages closely related to *Sodalis praecaptivus* (HS

32 strain) and suggest repeated acquisition of novel symbionts from the environment. Similar to

33 *Sodalis*, *Arsenophonus* endosymbionts also form both obligate endosymbiotic lineages co-

34 evolving with their hosts (Ornithomyini and Ornithoica groups) as well as possibly facultative

35 infections incongruent with the Hippoboscidae phylogeny. Finally, we reveal substantial

36 diversity of *Wolbachia* strains detected in Hippoboscidae samples falling into three

37 supergroups: A, B, and the most common F. Altogether, our results prove the associations

38 between Hippoboscoidea and their symbiotic bacteria to undergo surprisingly dynamic, yet

39 selective, evolutionary processes strongly shaped by repeated endosymbiont replacements.

40 Interestingly, obligate symbionts only originate from two endosymbiont genera, *Arsenophonus*

41 and *Sodalis*, suggesting that the host is either highly selective about its future obligate

42 symbionts or that these two lineages are the most competitive when establishing symbioses in

43 louse flies.

44 **Background**

45 Symbiotic associations are widespread among animals and bacteria and often considered to

46 undergo a common evolution as a holobiont (Zilber-Rosenberg & Rosenberg, 2008). The host

47 and symbiont are either fully dependent on each other for reproduction and survival (obligate

48 symbiosis) or not (facultative symbiosis), but in reality, there is a gradient of such interactions

49 (Moran, McCutcheon & Nakabachi, 2008). Any establishment of a symbiotic association

50 brings not only advantages, but also several challenges to both partners. Perhaps the most

51 crucial is that after entering the host, the endosymbiont genome tends to decay due to

52 population genetic processes affecting asexual organisms with small effective population sizes

53 (Moran, 1996) and the host is becoming dependent on such a degenerating symbiont (Koga et

54 al. 2007; Pais et al. 2008). Since symbionts are essential for the host, the host can try to escape

55 from this evolutionary 'rabbit hole' by an acquisition of novel symbionts or via endosymbiont

56 replacement and supplementation (Bennett & Moran, 2015). This phenomenon, known in

57 almost all insect symbiotic groups, was especially studied in the sap-feeding group Hemiptera

58 (Sudakaran, Kost & Kaltenpoth, 2017), while only few studies were performed from blood-

59 sucking groups.

60   Blood-sucking insects, living exclusively on vertebrate blood, such as sucking lice

61 (Allen et al. 2007; Hypša & Křížek 2007; Fukatsu et al. 2009; Allen et al. 2016), bed bugs

62 (Hypša & Aksoy, 1997; Hosokawa et al., 2010; Nikoh et al., 2014), kissing bugs (Ben-Yakir

63 1987; Beard et al. 1992; Hypša & Dale 1997; Šorfová et al. 2008; Pachebat et al. 2013), tsetse

64 flies (Aksoy, 1995; Dale & Maudlin, 1999), bat flies (Trowbridge, Dittmar & Whiting, 2006;

65 Hosokawa et al., 2012; Wilkinson et al., 2016), and louse flies (Trowbridge et al. 2006;

66 Nováková & Hypša 2007; Chrudimský et al. 2012) have established symbiotic associations

67 with bacteria from different lineages, mostly α-proteobacteria (Hosokawa et al., 2010) and γ-

68 proteobacteria (Aksoy 1995; Hypša & Aksoy 1997; Hypša & Dale 1997; Dale et al. 2006;

69 Allen et al. 2007; Hypša & Křížek 2007; Nováková & Hypša 2007; Chrudimský et al. 2012;

70 Hosokawa et al. 2012; Wilkinson et al. 2016). Obligate symbionts of these blood-sucking hosts

71 are hypothesized to supplement B-vitamins and cofactors missing from their blood diet or

72 present at too low concentration (Akman et al., 2002; Kirkness et al., 2010; Rio et al., 2012;

73 Nikoh et al., 2014; Nováková et al., 2015; Boyd et al., 2016, 2017), but experimental evidence

74 supporting this hypothesis is scarce (Hosokawa et al., 2010; Nikoh et al., 2014; Michalkova et

75 al., 2014; Snyder & Rio, 2015). The role played by facultative bacteria in blood-sucking hosts

76 is even less understood, with metabolic or protective function as the two main working

77 hypotheses (Geiger et al., 2005, 2007; Toh et al., 2006; Belda et al., 2010; Snyder et al., 2010;

78 Weiss et al., 2013).

79 Due to their medical importance, tsetse flies (Diptera, Glossinidae) belong to the most

80 frequently studied models of such symbioses (International Glossina Genome Initiative 2014).

81 They harbour three different symbiotic bacteria: obligate symbiont *Wigglesworthia glossinidia*

82 which is essential for the host survival (Pais et al., 2008), facultative symbiont *Sodalis*

83 *glossinidius* which was suggested to cooperate with *Wigglesworthia* on thiamine biosynthesis

84 (Belda et al., 2010), and reproductive manipulator *Wolbachia* (Pais et al., 2011). Considerable

85 amount of information has till now been accumulated on the distribution, genomics and

86 functions of these bacteria (Akman et al., 2002; Toh et al., 2006; Rio et al., 2012; Balmand et

87 al., 2013; Michalkova et al., 2014; Snyder & Rio, 2015). In contrast to our understanding of

88 tsetse fly symbioses, only scarce data are available on the symbioses in its closely related

89 groups. Apart from Glossinidae, the superfamily Hippoboscoidea includes additional three

90 families of obligatory blood-sucking flies, tightly associated with endosymbionts, namely

91 Nycteribiidae, Streblidae, and Hippoboscidae. Monophyly of Hippoboscoidea has been

92 confirmed by numerous studies (Nirmala, Hypša & Žurovec, 2001; Dittmar et al., 2006;

93 Petersen et al., 2007; Kutty et al., 2010), but its inner topology has not been fully resolved. The

94 monophyletic family Glossinidae is considered to be a sister group to the three remaining

95   families together designated as Pupipara (Petersen et al., 2007). The two groups associated

96   with bats probably form one branch, where Nycteribiidae seems to be monophyletic while

97   monophyly of Streblidae was not conclusively confirmed (Dittmar et al., 2006; Petersen et al.,

98   2007; Kutty et al., 2010). According to several studies, Hippoboscidae is regarded to be a

99   monophyletic group with not well-resolved exact position in the tree (Nirmala, Hypša &

100   Žurovec, 2001; Dittmar et al., 2006; Petersen et al., 2007). However, louse flies were also

101   shown to be paraphyletic in respect to bat flies (Dittmar et al., 2006; Kutty et al., 2010).

102   Nycteribiidae, Streblidae (bat flies), and Hippoboscidae (louse flies) are often

103   associated with *Arsenophonus* bacteria (Trowbridge, Dittmar & Whiting, 2006; Dale et al.,

104   2006; Nováková, Hypša & Moran, 2009; Morse et al., 2013; Duron et al., 2014). In some cases,

105   these symbionts form clades of obligate lineages coevolving with their hosts, but some of

106   *Arsenophonus* lineages are likely representing loosely associated facultative symbionts spread

107   horizontally across the population (Nováková, Hypša & Moran, 2009; Morse et al., 2013;

108   Duron et al., 2014). Bat flies and louse flies are also commonly infected with *Bartonella* spp.

109   (Halos et al., 2004; Morse et al., 2012b). *Wolbachia* infection was found in all Hippoboscoidea

110   groups (Pais et al., 2011; Hosokawa et al., 2012; Morse et al., 2012a; Nováková et al., 2015).

111   Moreover, several Hippoboscidae species were also found to harbour distinct lineages of

112   *Sodalis*-like bacteria (Dale et al. 2006; Nováková & Hypša 2007; Chrudimský et al. 2012)

113   likely representing similar facultative-obligatory gradient of symbioses as observed for

114   *Arsenophonus*.

115   Hippoboscoidea thus represent a group of blood-sucking insects with strikingly

116   dynamic symbioses. Obligate symbionts from *Arsenophonus* and *Sodalis* clades tend to come

117   and go, disrupting the almost flawless host-symbiont co-phylogenies often seen in insect-

118   bacteria systems. However, why are the endosymbiont replacements so common and what

119   keeps the symbiont consortia limited to the specific bacterial clades remains unknown. Tsetse

120   flies as medically important vectors of pathogens are undoubtedly the most studied

121   Hippoboscoidea lineage. However, their low species diversity (22 species), sister relationship

122   to all other clades, and host specificity to mammals, do not allow to draw any general

123   conclusions about the evolution of symbiosis in Hippoboscoidea. To fully understand the

124   symbiotic turn-over, more attention needs to be paid to the neglected Nycteriibidae, Streblidae,

125   and Hippoboscidae lineages. Here, using gene sequencing and draft genome data from all

126   involved partners, we present phylogenies of Hippoboscidae and their symbiont lineages and

127   try to untangle their relationship to the host. In particular, we ask if these are obligate co-

128   evolving lineages, facultative infections, or if they likely represent recent symbiont

129   replacements just re-starting the obligate relationship.

130   Methods

131   Sample collection and DNA isolation

132   Samples of louse flies were collected in seven countries (South Africa, Papua New Guinea,

133   Ecuador – Galapagos, Vietnam, France, Slovakia, and the Czech Republic; see Table S1 for

134   details), the single sample of bat fly was collected in the Czech Republic. All samples were

135   stored in 96% ethanol at -20°C. DNA was extracted using the QIAamp DNA Micro Kit

136   (Qiagen; Hilden, Germany) according to the manufacturer′s protocol. DNA quality was

137   verified using the Qubit High Sensitivity Kit (Invitrogen) and 1% agarose gel electrophoresis.

138

139   PCR, cloning, and sequencing

140   All DNA samples were used for amplification of three host genes (COI, 16S rRNA gene, EF)

141   and symbiont screening with 16S rRNA gene primers (Table S2). Ten *Wolbachia* positive

142   samples were used for MLST typing (*cox*A, *fbp*A, *fts*Z, *gat*B, *hcp*A; see Table S2). PCR

143   reaction was performed under standard conditions using High Fidelity PCR Enzyme Mix

144   (Thermo Scientific) and Hot Start Tag DNA Polymerase (Qiagen) according to the

145   manufacturer's protocol. PCR products were analysed using 1% agarose gel electrophoresis

146   and all symbiont 16S rDNA products were cloned into pGEM®–T Easy vector (Promega)

147   according to the manufacturer's protocol. Inserts from selected colonies were amplified using

148   T7 and SP6 primers or isolated from plasmids using the Plasmid Miniprep Spin Kit (Jetquick).

149   Sanger sequencing was performed by an ABI Automatic Sequencer 3730XL (Macrogen Inc.,

150   Geumchun-gu-Seoul, Korea) or ABI Prism 310 Sequencer (SEQme, Dobříš, the Czech

151   Republic).

152   In addition to sequencing, we also included in our analyses genomic data of

153   *Melophagus ovinus* (Nováková et al., 2015), *Lipoptena cervi* (Nováková et al., 2016),

154   *Ornithomya biloba*, and *Crataerina pallida* (E. Šochová unpublished data) as well as their

155   endosymbionts (see Table S1).

156   Although there is MLST available for *Arsenophonus* bacteria (Duron, Wilkes & Hurst,

157   2010), we were not successful in amplifying these genes.

158

159   **Alignments and phylogenetic analyses**

160   The assemblies of raw sequences were performed in Geneious v8.1.7 (Kearse et al., 2012).

161   Datasets were composed of the assembled sequences, extracted genomic sequences, sequences

162   downloaded from GenBank (see Supplemental Table S4) or the *Wolbachia* MLST database.

163   The sequences were aligned with Mafft v7.017 (Katoh, 2002; Katoh, Asimenos & Toh, 2009)

164   implemented in Geneious using an E-INS-i algorithm with default parameters. The alignments

165   were not trimmed as trimming resulted in massive loss of informative position. Phylogenetic

166   analyses were carried out using maximum likelihood (ML) in PhyML v3.0 (Guindon &

167   Gascuel, 2003; Guindon et al., 2009) and Bayesian inference (BI) in MrBayes v3.1.2

168   (Huelsenbeck & Ronquist, 2001). The *GTR+I+Γ* evolutionary model was selected in

169   jModelTest (Posada, 2009) according to the Akaike Information Criterion (AIC). The subtree

170   prunning and regrafting (SPR) tree search algorithm and 100 bootstrap pseudoreplicates were

171   used in the ML analyses. BI runs were carried out for 10 million generations with default

172   parameters, and Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/) was used for

173   convergence and burn-in examination. Phylogenetic trees were visualised and rooted in

174   FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and their final graphical adjustments

175   were performed in Inkscape v0.91 (https://inkscape.org/en/).

176   Host phylogeny was reconstructed using single-gene analyses and a concatenated

177   matrix of three genes (mitochondrial 16S rRNA, mitochondrial cytochrome oxidase I, and

178   nuclear elongation factor). Concatenation of genes was performed in Phyutility 2.2.6 (Smith &

179   Dunn, 2008). Phylogenetic trees were inferred for all species from the Hippoboscoidea

180   superfamily, as well as for smaller datasets comprising only Hippoboscidae species. This

181   approach was employed to reveal possible artefacts resulting from missing data and poor taxon-

182   sampling (e.g. short, ~ 360 bp, sequences of COI available for Streblidae and Nycteribiidae).

183

184   **Mitochondrial genomes**

185   Problems with reconstruction of host phylogeny based on mitochondrial genes (16S and COI)

186   lead us to assemble mitochondrial genomes of four main louse fly lineages. Contigs of

187   mitochondrial genomes were identified in genomic data of *M. ovinus*, *L. cervi*, *O. biloba*, and

188   *C. pallida* using BLASTn and tBLASTn searches (Altschul et al., 1990). Open reading frame

189   identification and preliminary annotations were performed using NCBI BlastSearch in

190   Geneious. For identification of Numts, raw sequences were mapped to mitochondrial data

191   using Bowtie v2.2.3 (Langmead & Salzberg, 2012). Web annotation server MITOS

192   (http://mitos.bioinf.uni-leipzig.de/) was used for final annotation of proteins and rRNA/tRNA

193   genes. We selected 15 mitochondrial genes (Table S4) present in all included taxa for

194   phylogenetic inference as described above.

195

Results

Phylogenetic data

198 We obtained 134 host sequences: 31 sequences of 16S rRNA of 370 - 545 bp, 47 sequences of

199 EF of 207 - 922 bp, and 56 sequences of COI of 299 - 1,522 bp; and 70 symbiont 16S rRNA

200 sequences of 777 - 1,589 bp. We also assembled and annotated 4 host mitochondrial genomes

201 of 15,975 – 16,445 bp. For more details see Supplemental Table S3. All raw sequences can be

202 found online in Supplemental Data S1 (their description is included in Supplemental Table S6).

203

Hipboboscidae phylogeny

205 We reconstructed host phylogeny using three markers: 16S rRNA, EF and COI; as well as

206 mitochondrial genomes. Our analyses of draft genome data revealed that all analysed

207 mitochondrial genomes of louse flies are also present as Numts (nuclear mitochondrial DNA)

208 on the host chromosomes, especially the COI gene often used for phylogenetic analyses. The

209 taxonomically restricted mitochondrial genome matrix verified monophyly of Hippoboscoidea

210 (Supplemental Figure Fig. S1). Our three-gene dataset yielded only partially resolved and

211 unstable inner Hippoboscoidea phylogeny. Glossinidae and Nycteribiidae formed a well-

212 defined monophyletic groups (only ML analysis of COI did not confirm monophyly of

213 Nycteribiidae and also did not resolve its relationship to Streblidae), but monophyly of

214 Hippoboscidae and Streblidae was not well supported and different genes/analyses frequently

215 inferred contradictory topologies. Within Hippoboscidae, the position of the Hippoboscinae

216 group and the genus *Ornithoica* were the most problematic (Fig. 1, Supplemental Figures Fig.

217 S2-8).

218

Arsenophonus and *Sodalis* phylogenies

220   In total, 72 endosymbiont 16S rRNA genes were sequenced in this study and six additional

221   sequences of this gene were mined from our draft genomic data: four of *Arsenophonus*, one of

222   *Sodalis*, and one of *Wolbachia*. Twenty eight symbionts were identified as members of the

223   genus *Arsenophonus*, 13 symbionts were the most similar to *Sodalis*-allied species, and 31

224   sequences were of *Wolbachia* origin. Despite of cloning, we did not obtain any sequences of

225   *Bartonella* reported to occur in some Hippoboscoidea. Moreover, using only phylogenetic

226   approach, we would not be able to decide whether *Bartonella*-Hippoboscidae interaction is

227   mutualistic or pathogenic, therefore *Bartonella* symbiosis is not in the scope of this manuscript.

228   Putative assignment to the obligate or likely facultative symbiont categories was based on GC

229   content of their 16S rRNA gene and genomic data available (Supplemental Table S3), branch

230   length, and the phylogenetic analyses.

231   Phylogenetic analyses of the genus *Arsenophonus* based on 16S rDNA sequences

232   revealed several distinct clades of likely obligate *Arsenophonus* species congruent with their

233   host phylogeny, partially within the Nycteribiidae, Streblidae, and several Hippoboscidae

234   lineages (Fig. 2, Supplemental Figures Fig. S9 and Fig. S10). However, it is important to note

235   that these clades do not form a single monophyletic clade of co-diverging symbionts, but rather

236   several separate lineages. Within Hippoboscidae, the *Arsenophonus* sequences from the

237   Ornithomyini group form a monophyletic clade congruent with Ornithomyini phylogeny. With

238   the exception of *Arsenophonus* symbiont of *Crataerina* spp. which was probably recently

239   replaced by another *Arsenophonus* bacteria. Other obligate *Arsenophonus* lineages were

240   detected in the genera *Lipoptena*, *Melophagus*, and *Ornithoica*. All other *Arsenophonus*

241   sequences from the Hippoboscidae either represent facultative symbionts or putatively obligate

242   symbioses which are impossible to reliably detect by phylogenetic methods (but see the

243   discussion for *Hippobosca* sp.).

244   Most of the putatively facultative endosymbionts of the Hippoboscidae typically

245 possess short branches and are also related with the previously described species *Arsenophonus*

246 *arthropodicus* and *Arsenophonus nasoniae*. Interestingly, both obligate and likely facultative

247 lineages were detected from several species, e.g. *Ornithomya biloba*, *Ornithomya avicularia*,

248 and *Ornithomya fringillina* (Fig. 2). Phylogenetic analyses including symbionts from the

249 genera *Nycterophylia* and *Trichobius* did not clearly place them into the *Arsenophonus* genus.

250 Rather, they likely represent closely related lineages to the *Arsenophonus* clade as their position

251 was unstable and changed with different taxon samplings and methods.

252   Within *Sodalis*, the phylogenetic reconstruction revealed a putatively obligate

253 endosymbiont from the tribe Olfersini, including the genera *Pseudolynchia* and *Icosta*, and

254 several facultative lineages. However, co-evolution with *Icosta* sp. seems to be imperfect and

255 does not strictly follow the host phylogeny (Fig. 3).

256

257 ***Wolbachia* MLST analysis**

258 In *Wolbachia*, the 16S rDNA sequences were used only for an approximate supergroup

259 determination (Fig. 4). The MLST analysis was performed with ten selected species (one of

260 them was obtained from genomic data of *O. biloba*; see Table S3). Overall prevalence of

261 *Wolbachia* in louse flies is 54.55 %; 30 positive individuals out of 55 diagnosed. The

262 supergroup A was detected from 4 species (4 individuals), the supergroup B from 5 species (9

263 individuals), and the supergroup F from 7 species (17 individuals) (Fig. 4). Additionally,

264 *Nycteribia kolenatii* (one individual) was infected with the supergroup F.

265

266 **Discussion**

267 **Hippoboscidae phylogeny: an unfinished portrait**

268 Although closely related to the medically important tsetse flies, the other hippoboscoids have

269 only rarely been studied and their phylogeny is still unclear. Based on our concatenated matrix,

270 we obtained the topology which to some extent resembles the one presented Petersen et al.

271 (2007), although with slightly different taxon sampling (Fig. 1; Supplemental Figure Fig S2).

272 However, our three single-gene datasets implied only poor phylogenetic signal available

273 carried by the hippoboscoid sequences. Therefore, we took an advantage of the four complete

274 mitochondrial genomes reconstructed in this study to test the reliability of the previous

275 phylogenetic reconstructions. The phylogenetic reconstruction based on the mitochondrial

276 matrix correspond to the three-gene concatenated matrix phylogeny suggesting that

277 mitochondrial genomes would be valuable for further phylogenetic analyses of this group (Fig.

278 1; Supplemental figure Fig. S1). According to our results, Glossinidae, Nycteribiidae and

279 Hippoboscidae were retained as monophyletic groups, but monophyly of Streblidae was not

280 supported using the complete matrix (Supplemental Figure Fig. S2). Streblidae lineage appears

281 to be paraphyletic with respect to Nycteribiidae and clusters into two groups, the Old World

282 and the New World species, as previously reported (Dittmar et al., 2006; Kutty et al., 2010).

283 Within Hippoboscidae, the groups Lipopteninae, Hippoboscinae, Ornithomyini and Olfersini

284 (nomenclature was adopted from Petersen et al. (2007)) are well-defined and monophyletic,

285 but their exact relationships are still not clear. The most problematic taxa are Hippoboscinae

286 and also the genus *Ornithoica* with their positions depending on the used genes/analyses (Fig.

287 1; Supplemental figures Fig. S2-8). A possible explanation for these inconsistencies in the

288 topologies can be a hypothetical rapid radiation from the ancestor of Hippoboscoidea group

289 into main subfamilies of Hippoboscidae leaving in the sequences only very weak phylogenetic

290 signal for this period of Hippoboscidae evolution. The most difficulties in reconstructing

291 Hippoboscoidea phylogeny is caused by missing data (only short sequences of COI are

292 available especially for Nycteribiidae and Streblidae in the GenBank; Supplemental Figure Fig.

293   S3). Moreover, COI phylogenies are known to be affected by numerous pseudogenes called

294   Numts (Black IV & Bernhardt 2009). The Numts, we found to be common in louse fly

295   genomes, can thus also contribute to the intricacy of presented phylogenies. On the other hand,

296   EF seems to provide plausible phylogenetic information (Supplemental Figure Fig. S4). The

297   biggest drawback of this marker however lies in the data availability in public databases,

298   restricting an appropriate taxon sampling for the Hippoboscoidea superfamily.

299

Hidden endosymbiont diversity within the Hippoboscidae family

301   Among the three most commonly detected Hippoboscidae endosymbionts, attention has been

302   predominantly paid to *Arsenophonus* as the supposedly most common obligate endosymbiont

303   of this group. Our data show that several different lineages of *Arsenophonus* have established

304   the symbiotic lifestyle within Hippoboscidae (Fig. 2). According to our results supported by

305   genomic data, there are at least four lineages of likely obligate endosymbionts: *Arsenophonus*

306   in Ornithomyini (genomes of *Arsenophonus* from *Ornithomya biloba* and *Crataerina pallida*

307   will be published elsewhere), *Arsenophonus* in *Ornithoica* spp., previously described

308   *Arsenophonus melophagi* (Nováková et al., 2015) and *Arsenophonus lipopteni* (Nováková et

309   al., 2016). All these possess reduced genomes with low GC content as a typical feature of

310   obligate endosymbionts (McCutcheon & Moran, 2012). Interestingly, within Ornithomyini, the

311   original obligate *Arsenophonus* endosymbiont of *Crataerina* spp. was recently replaced by

312   another *Arsenophonus* bacterium with ongoing genome reduction (E. Šochová unpublished

313   data). Apart from these potentially obligate lineages, there are other hippoboscid associated

314   *Arsenophonus* bacteria distributed in the phylogenetic tree among *Arsenophonus*

315   endosymbionts with likely facultative or free-living lifestyle (Supplemental Figure Fig. S10).

316   This pattern suggests *Arsenophonus* is likely being repeatedly acquired from the environment.

317   It has been hypothesized that obligate endosymbionts often evolve from facultative symbionts

318   which are no longer capable of horizontal transmission between the hosts (Moran, McCutcheon

319   & Nakabachi, 2008). Due to their recent change of lifestyle, endosymbionts with an ongoing

320   genome reduction in many ways resemble facultative symbionts, e.g. their positions in

321   phylogenetic trees are not stable and differ with the analysis method and taxon sampling (Fig.

322   2, Supplemental Figures Fig. S9 and S10). Such nascent stage of endosymbiosis was indicated

323   for the obligate *Arsenophonus* endosymbiont of *C. pallida* (E. Šochová unpublished data) and

324   similar results can be expected for *Arsenophonus* endosymbionts of *Hippobosca* species.

325

326   Within bat flies, we found obligate *Arsenophonus* lineages in both Nycteribiidae and Streblidae

327   as well as several presumably facultative *Arsenophonus* infections in both groups

328   (Supplemental Figures Fig. S9 and S10). Similar results were reported in several previous

329   studies (Morse et al., 2013; Duron et al., 2014; Wilkinson et al., 2016). Members of the

330   *Arsenophonus* clade were also reported from Nycterophyliinae and Trichobiinae (Streblidae)

331   (Morse et al., 2012a) and *Cyclopodia dubia* (Nycteribiidae) (Wilkinson et al., 2016). However,

332   our results do not support their placement within the clade, as these sequences were attracted

333   by the long branches in the ML analyses. The endosymbiont of Nycterophyliinae and

334   Trichobiinae probably represents an ancient lineage closely related to *Arsenophonus* clade

335   (Supplemental Figure Fig. S9) while the endosymbiont of *Cyclopodia dubia* is more likely

336   related with *Pectobacterium* spp.; therefore, we excluded this bacterium from our further

337   analyses. These findings indicate that bat flies established the endosymbiotic lifestyle several

338   times independently with at least three bacterial genera.

339

340   In contrast to *Arsenophonus*, only a few studies reported *Sodalis*-like endosymbiotic bacteria

341   from Hippoboscidae (Nováková & Hypša 2007; Chrudimský et al. 2012; Nováková et al.

342   2015). Dale et al. (2006) detected a putative obligate endosymbiont from *Pseudolynchia*

343 *canariensis* which was suggested to represent *Sodalis* bacterium. We detected this symbiont in

344 several members of the Olfersini group and according to our results, it is obligate *Sodalis*-like

345 endosymbiont forming a monophyletic clade, but its congruence with the Olfersini phylogeny

346 is somewhat imperfect (Fig. 3). This incongruence might be a consequence of phylogenetic

347 artefacts likely affecting long branches of *Sodalis* symbionts from *Icosta*. Similar to

348 *Arsenophonus*, *Sodalis* bacteria also establish possible facultative associations, e. g. with

349 *Melophagus ovinus* (Chrudimský et al., 2012; Nováková et al., 2015), *Ornithomya avicularia*

350 (Chrudimský et al., 2012) or *Ornithomya biloba* (this study). *Sodalis* endosymbiont from

351 *Crataerina melbae* was suggested to be obligate (Nováková & Hypša 2007), but our study did

352 not support this hypothesis since it clusters with free-living *Sodalis praecaptivus*. Interestingly,

353 *Sodalis* endosymbiont of *Microlynchia galapagoensis* was inferred to be closely related to

354 *Sodalis*-like co-symbiont of *Cinara cedri*, which underwent rapid genome deterioration after a

355 replacement of former co-symbiont (Meseguer et al., 2017). These results suggest that there

356 are several loosely associated lineages of *Sodalis* bacteria in louse flies. On one hand, the

357 endosymbiont of *Microlynchia galapagoensis* probably represents a separate (or ancient)

358 *Sodalis* infection, but on the other hand, other *Sodalis* infections seem to be repeatedly acquired

359 from the environment as implied by their relationship to e.g. *Sodalis praecaptivus* (Clayton et

360 al., 2012) (Fig. 3).

361

362 Coinfections of obligate and facultative *Arsenophonus* strains in Hippoboscidae (or potentially

363 *Sodalis* in Olfersini) are extremely difficult to recognize using only PCR-acquired 16S rRNA

364 gene. Facultative endosymbionts retain several copies of this gene and thus their 16S rRNA

365 tend to be amplified more likely in PCR than from reduced obligate endosymbionts due to its

366 higher copy number and lower frequency of mutations in primer binding sites. Even though

367 there is a MLST available for *Arsenophonus* bacteria (Duron, Wilkes & Hurst, 2010), it was

368 shown that it is effective only partially (Duron et al., 2014). Since our data are probably also

369 influenced by this setback, we do not speculate which of the detected potentially facultative

370 *Arsenophonus* lineages represent source of 'ancestors' for several distinct obligate lineages or

371 which of them were involved in the recent replacement scenario. However, the

372 replacement/independent-origin scenario is well illustrated by endosymbionts from Olfersini

373 (Fig. 2, Fig. 3).

374

375 To complement the picture of Hippoboscidae endosymbiosis, we also reconstructed *Wolbachia*

376 evolution. We found three different supergroups: A, B and F (see Table S3). Apparently, there

377 is no coevolution between *Wolbachia* and Hippoboscidae hosts suggesting horizontal

378 transmission between species (Fig. 4) as common for this bacterium (Schilthuizen &

379 Stouthamer, 1997; Gerth et al., 2014). Since *Wolbachia* seems to be one of the most common

380 donors of genes horizontally transferred to insect genomes, including tsetse flies (Husník et al.

381 2013; Brelsfoard et al. 2014; Sloan et al. 2014), we cannot rule out that some of *Wolbachia*

382 sequences detected in this study represent HGT insertions into the respective host genomes.

383 The biological role of *Wolbachia* in Hippoboscidae was never examined in spite of its relatively

384 high prevalence in this host group (55%). The F supergroup was detected as the most frequent

385 lineage in Hippoboscidae which is congruent with its common presence in blood-sucking

386 insects such as Streblidae (Morse et al., 2012a), Nycteribiidae (Hosokawa et al., 2012),

387 Amblycera (Covacin & Barker, 2007), and Cimicidae (Hosokawa et al., 2010; Nikoh et al.,

388 2014).

389

390 Besides the three main Hippoboscidae symbionts we paid attention to, *Bartonella* spp. that are

391 also widespread among louse flies and bat flies. The infection seems to be fixed only in

392 *Melophagus ovinus* suggesting a mutualistic relationship (Halos et al., 2004), but additional

393 functional data are needed to confirm this hypothesis (Nováková et al., 2015). Nevertheless,

394 deer ked and sheep ked are also suspected of vectoring bartonellosis (Maggi et al., 2009; de

395 Bruin et al., 2015). According to the recent findings, *Bartonella* spp. used to be originally gut

396 symbionts which adapted to pathogenicity (Hid Segers et al., 2016; Neuvonen et al., 2016).

397

What is behind dynamics of Hippoboscidae-symbiont associations?

399 According to our results, symbiosis in the Hippoboscidae group is very dynamic and influenced

400 by frequent symbiont replacements. *Arsenophonus* and *Sodalis* infections seem to be the best

401 resources for endosymbiotic counterparts, but it remains unclear why just these two genera.

402 Both are endowed with several features of free-living/pathogenic bacteria enabling them to

403 enter new host which can be crucial in establishing novel symbiotic association. *Sodalis*

404 *glossinidius* possesses modified outer membrane protein (OmpA) which is playing an

405 important role in the interaction with the host immune system (Weiss et al., 2008; Weiss, Maltz

406 & Aksoy, 2012). Both *Sodalis* and *Arsenophonus* bacteria retain genes for the type III secretion

407 system (Dale et al., 2001; Wilkes et al., 2010; Chrudimský et al., 2012; Oakeson et al., 2014)

408 allowing pathogenic bacteria to invade eukaryotic cells. Moreover, several strains of these

409 bacteria are cultivable under laboratory conditions (Hypša & Dale 1997; Dale & Maudlin 1999;

410 Dale et al. 2006; Darby et al. 2010; Chrudimský et al. 2012; Chari et al. 2015) suggesting that

411 they should be able to survive horizontal transmission. For instance, *Arsenophonus nasoniae*

412 is able to spread by horizontal transfer between species (Duron, Wilkes & Hurst, 2010), while

413 *Sodalis*-allied bacteria have several times successfully replaced ancient symbionts (Conord et

414 al., 2008; Koga et al., 2013; Meseguer et al., 2017).

415

416 Whereas the facultative endosymbionts of Hippoboscoidea are widespread in numerous types

417 of tissues such as milk glands, bacteriome, haemolymph, gut, fat body, and reproductive organs

418 (Dale & Maudlin, 1999; Dale et al., 2006; Balmand et al., 2013; Nováková et al., 2015), the

419 obligate endosymbionts are restricted to the bacteriome and milk glands (Aksoy, 1995; Attardo

420 et al., 2008; Balmand et al., 2013; Morse et al., 2013; Nováková et al., 2015). Entering the milk

421 glands ensures vertical transmission of facultative endosymbiont to progeny and better

422 establishment of the infection. Vertical transmission also enables the endosymbiont to hitch-

423 hike with the obligate endosymbiont and because the obligate endosymbiont is inevitably

424 degenerating (Moran, 1996; Wernegreen, 2002), the new co-symbiont can eventually replace

425 it if needed. For instance, *Sodalis melophagi* was shown to appear in both milk glands and

426 bacteriome and to code for the same full set of B-vitamin pathways (including in addition the

427 thiamine pathway) as the obligate endosymbiont *Arsenophonus melophagi* (Nováková et al.,

428 2015). This suggests that it could be potentially capable of shifting from facultative to

429 obligatory lifestyle and replace the *Arsenophonus melophagi* endosymbiont.

430

431 We suggest that the complex taxonomic structure of the symbiosis in Hippoboscoidea can be

432 result of multiple replacements, similar to that already suggested for the evolution of symbiosis

433 in *Columbicola* lice (Smith et al., 2013) or mealybugs (Husník & McCutcheon 2016). Based

434 on the arrangement of the current symbioses in various species of Pupipara, the ancestral

435 endosymbiont was likely either an *Arsenophonus* or *Sodalis* bacterium (given our finding of

436 the potential obligate *Sodalis* lineage in Olfersini). In the course of Pupipara evolution and

437 speciation, this symbiont was repeatedly replaced by different *Arsenophonus* (or *Sodalis* in

438 Olfersini if not ancestral) lineages, as indicated by the lack of phylogenetic congruence and

439 differences in genome reduction, gene order, and GC content in separate *Arsenophonus*

440 lineages (Nováková et al. 2015, 2016; E. Šochová unpublished data). This genomic diversity

441 across the *Arsenophonus* bacteria from distinct Hippoboscidae thus likely reflects their

442 different age correlating with the level of genome reduction in symbiotic bacteria.

443

Conclusions

Despite the considerable ecological and geographical variability, the Hippoboscoidea families surprisingly share some aspects of their association with symbiotic bacteria. Particularly, they show high affinity to two bacterial genera, *Arsenophonus* and *Sodalis*. This affinity is not only reflected by frequent occurrence of the bacteria but mainly by their multiple independent acquisitions. Comparisons between the hippoboscid and bacterial phylogenies indicate several independent origins of the symbiosis, although more precise evolutionary reconstruction is still hampered by the uncertainties in hippoboscid phylogenies.

Acknowledgement

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788

Figure 1 Host phylogeny derived from concatenation of three genes: 16S rRNA, EF, and COI. The phylogeny was reconstructed by BI analysis. Posterior probabilities and bootstrap support are printed upon branches, respectively (asterisk was used for very low or missing bootstrap branch support)**.** Taxa labelled with voucher are newly sequenced in this study. Genomic COI sequences are labelled with rRNA. Three smaller trees on the top of the figure represent outlines of three separate phylogenetic trees based on BI analyses of 16S rRNA, EF, and COI genes**.** Full versions of these phylogenies are included in Supplemental Figures (Fig. S6-8). Three main families of Hippoboscidae are colour coded: yellow for Lipopteninae (one group), brown for Hippoboscinae (one group), and orange for Ornithomiinae (three groups). Colour squares label branches where are placed main Hippoboscidae groups. This labelling corresponds with labelling of branches at smaller outlines, which are in addition to this highlighted with the same colour. All host trees are included in Supplemental Figures (Fig. S1-8).

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16S

EF

COI

1/100
Ornithoica sp. 1 voucher P16
Ornithoica turdi voucher K16N
0,92/78
Ornithoica turdi voucher K14N
Ornithoica turdi voucher K15N
Ornithoica turdi voucher K17N
Ornithoica turdi voucher OT2

Ornithomya avicularia voucher Z3
Ornithomya avicularia voucher K26N
1/100
Ornithomya avicularia voucher K24N
Ornithomya avicularia voucher K25N
Ornithomya avicularia voucher K29N
Ornithomya avicularia
0,99/92
Ornithomya avicularia voucher OA
Ornithomya chloropus
1/100
1/100
Stenepteryx hirundinis
0,91/55
Ornithomya biloba
0,89/69
1/100
Ornithomya biloba voucher OB
Ornithomya biloba voucher K40N
1/76
1/100
Ornithomya fringillina voucher K18N
Ornithomya fringillina voucher K27N
1/100
Crataerina melbae voucher CM
1/99
Crataerina pallida
Crataerina pallida voucher CP

0,93/52

1/100
Melophagus ovinus
Melophagus ovinus voucher MO
1/90
1/100
Lipoptena cervi
1/100
Lipoptena cervi voucher JE43
Lipoptena fortisetosa voucher FH16

0,97/59
1/100
Hippobosca rufipes
Hippobosca equina voucher HE1

1/95

0,87/*
Ortholfersia minuta
0,53/55
Microlynchia galapagoensis voucher MG1
0,69/55
Pseudolynchia sp. voucher E1
1/98
1/95
Pseudolynchia sp. voucher F5
1/100
Pseudolynchia sp. voucher U2
Pseudolynchia sp. voucher P24
1/100
Pseudolynchia canariensis
1/78
Icosta sp. 2 voucher P5
Icosta sp. 3 voucher A2
0,84/57
1/100
Icosta sp. 1 voucher G1
0,89/71
Icosta sp. 1 voucher G9
Icosta sp. 4 voucher WAN

0,56/*
Lucilia sericata
1/62
Sarcophaga crassipalpis
0,84/*
Protophormia terraenovae
Musca domestica
1/100
Pollenia rudis

0.03

Figure 2 16S rRNA phylogeny of *Arsenophonus* in Hippoboscidae inferred by BI analysis.

Posterior probabilities and bootstrap support are printed upon branches, respectively (asterisk was used for very low or missing bootstrap branch support). Taxa labelled with voucher are newly sequenced in this study. Genomic sequences are labelled with rRNA. Taxa in dark purple represent Arsenophonus bacteria which genome was sequenced. Numbers behind these taxa correspond to their GC content of 16S rRNA, GC content of genome, and genome size, respectively. Numbers behind other taxa correspond to GC content of their 16S rRNA. Smaller picture on the right side represents host phylogeny to which symbiont phylogeny was compared. Red lineages correspond to obligate symbionts while orange lineage is symbiont of recent origin. Blue A represent likely facultative *Arsenophonus* infection. To achieve this, we also used the information available on *gro*EL gene by Morse et al. (2013) and Duron et al. (2014). Phylogenetic reconstructions of *Arsenophonus* of entire Hippoboscoidea and all *Arsenophonus* bacteria are included in Supplemental Figures (Fig. S9 and Fig. S10).

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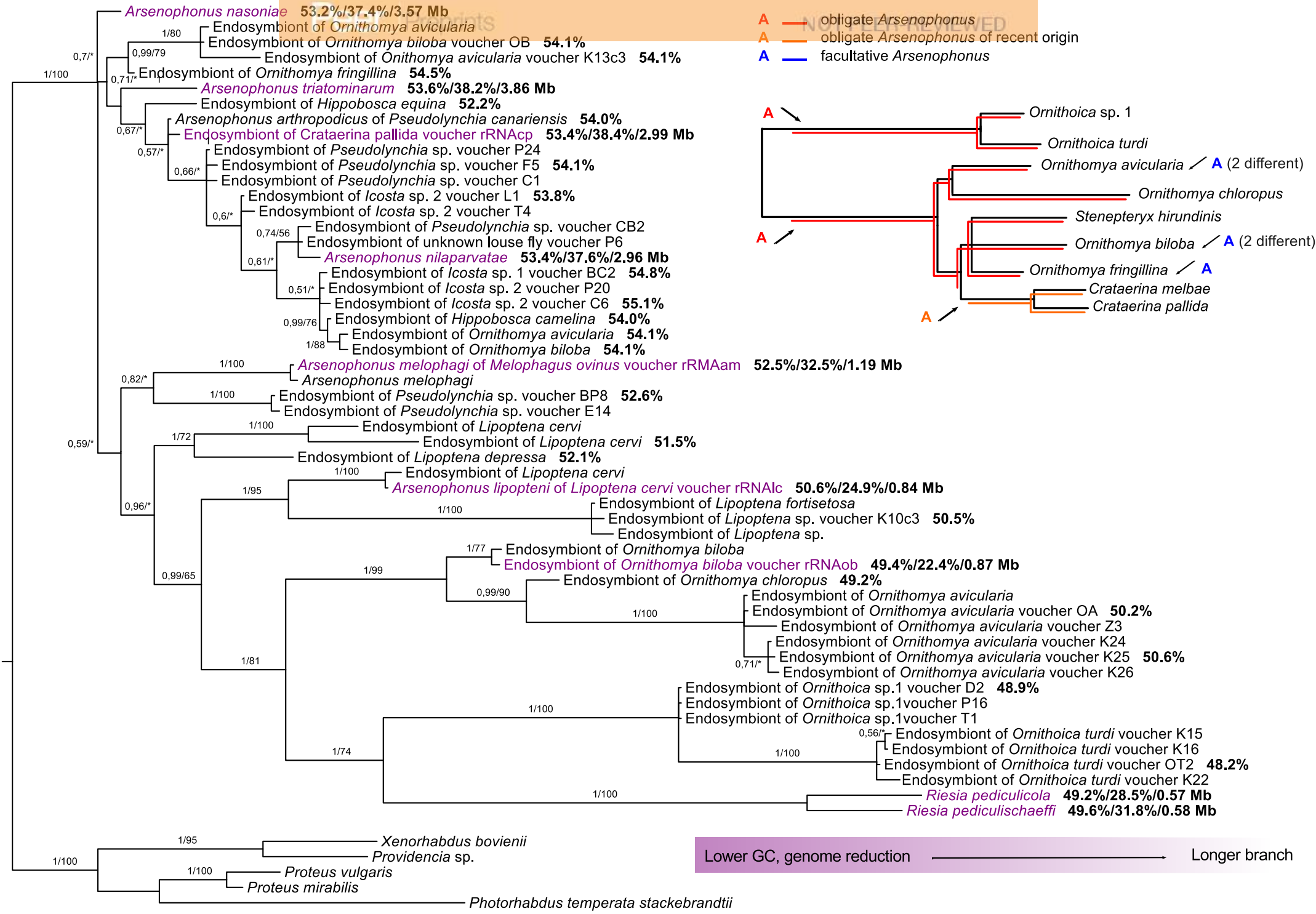
Figure 3 16S rRNA phylogeny of *Sodalis* in Hippoboscidae inferred by BI analysis. Posterior probabilities and bootstrap support are printed upon branches, respectively (asterisk was used for very low or missing bootstrap branch support)**.** Taxa labelled with voucher are newly sequenced in this study. Taxa in dark purple represent *Sodalis*-like bacteria which genome was sequenced. Numbers behind these taxa correspond to their GC content of 16S rRNA, GC content of genome, and genome size, respectively. Numbers behind other taxa correspond to GC content of their 16S rRNA. Red lineages correspond to obligate symbionts while orange lineage is symbiont of recent origin. Red dashed line shows that co-evolution between *Icosta* spp. and their obligate endosymbiont imperfect.

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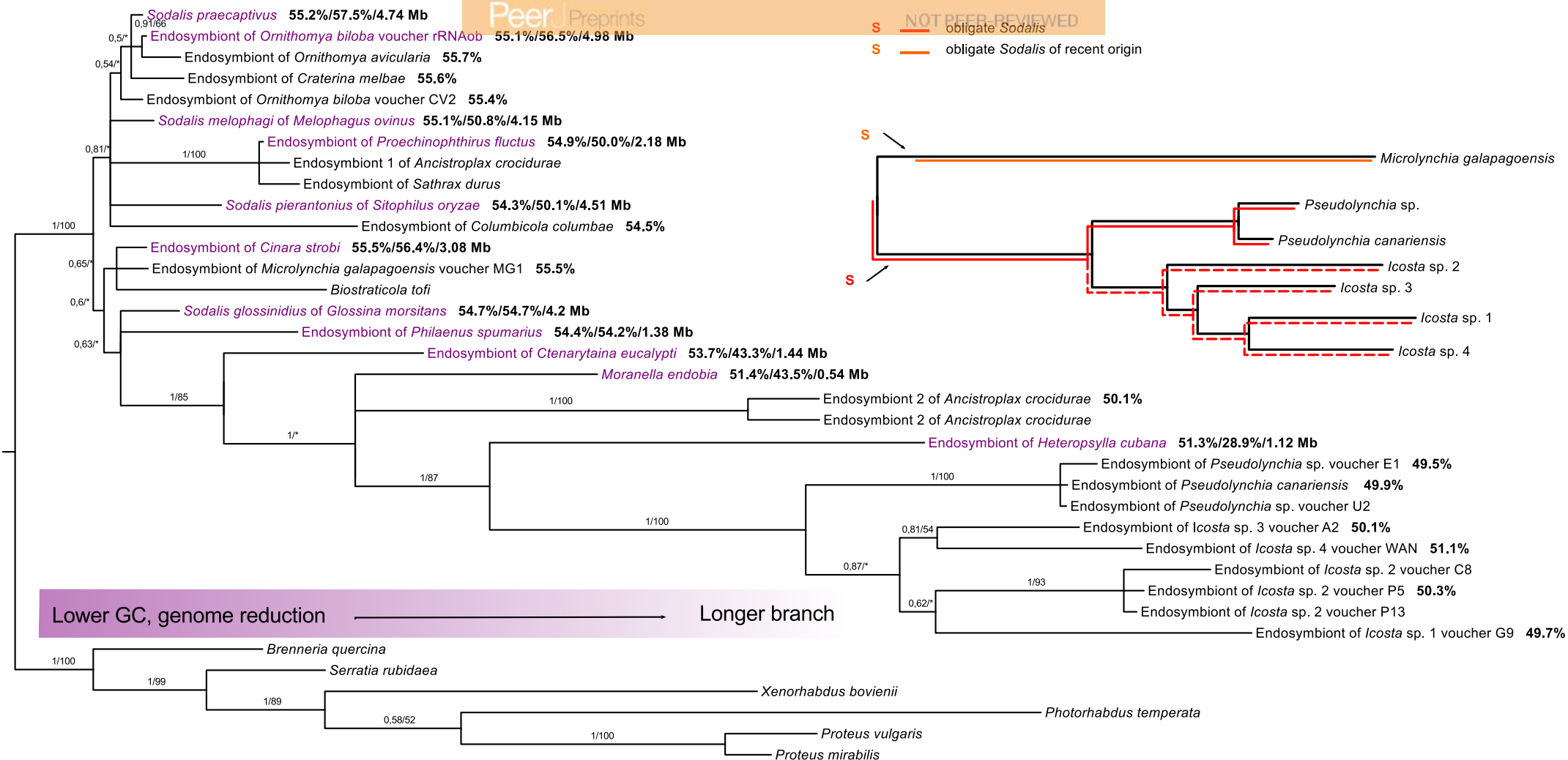
Figure 4 *Wolbachia* phylogeny inferred from 16S rRNA and MLST genes by BI analysis. Posterior

probabilities and bootstrap support are printed upon branches, respectively (asterisk was used for very

low or missing bootstrap branch support)**.** Colour letters upon branches correspond to *Wolbachia*

supergroups. Taxa in red represent *Wolbachia* bacteria from Hippoboscidae and Nycteribidae which

are newly sequenced in this study. Taxa labelled with # in the 16S tree represent taxa which were used

for the MLST analysis. *Wolbachia* from *O. biloba*, which was obtained from genomic data, is labelled

with rRNAob. Supergroup E was used for rooting both trees.

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16S

A

Wolbachia of *Icosta* sp. 4 voucher WAN
Wolbachia of *Ornithomya biloba* voucher rRNAob
Wolbachia of *Crataerina pallida* voucher CP
0,99/71

Wolbachia of *Icosta* sp. 3 voucher A2

1/100

B

Wolbachia of *Lipoptena fortisetosa* voucher FH16
Wolbachia of *Microlynchia galapagoensis* voucher MG1
0,99/93

Wolbachia of *Icosta* sp. 2 voucher P5
Wolbachia of *Icosta* sp. 2 voucher P20
0,91/59

Wolbachia of *Icosta* sp. 2 voucher C7
Wolbachia of *Icosta* sp. 2 voucher L1
0,99/78

1/89

Wolbachia of *Tribolium confusum*
Wolbachia of *Pseudolynchia canariensis* Type 2
0,73/58

Wolbachia of *Ornithomya biloba* voucher CV2

0,8/62

Wolbachia of *Ornithomya avicularia* voucher Z2

0,88/61

F

Wolbachia of *Ornithoica turdi* voucher OT2
Wolbachia of *Icosta* sp. 2 voucher CF2

1/95

Wolbachia of *Icosta* sp. 1 voucher G1
Wolbachia of *Pseudolynchia* sp. voucher F5
Wolbachia of *Pseudolynchia* sp. voucher S1
Wolbachia of *Pseudolynchia canariensis* Type 1
Wolbachia of *Pseudolynchia* sp. voucher C1
Wolbachia of *Cimex lectularius*

0,55/56

Wolbachia of *Pseudolynchia* sp. voucher C2

0,79/*

Wolbachia of *Lipoptena cervi* voucher JE43
Wolbachia of *Icosta* sp. 1 voucher P8
0,99/69

Wolbachia of *Pseudolynchia* sp. voucher CB2
Wolbachia of unknown louse fly voucher P6
Wolbachia of *Pseudolynchia* sp. voucher E14
Wolbachia of *Icosta* sp. 2 voucher L6
0,87/58

Wolbachia of *Melophagus ovinus* voucher MO
Wolbachia of *Pseudolynchia* sp. voucher P24
0,83/*

Wolbachia of *Pseudolynchia* sp. voucher C4
Wolbachia of *Pseudolynchia* sp. voucher E1
0,61/*

E

Wolbachia of *Folsomia candida*
Wolbachia of *Mesaphorura macrochaeta*
1/100

0.007

MLST

A

Wolbachia of *Crataerina pallida* voucher CP
Wolbachia of *Muscidifurax uniraptor*
0,97/64

1/98

Wolbachia of *Drosophila simulans*
1/84

Wolbachia of *Icosta* sp. 4 voucher WAN
0,99/58

Wolbachia of *Drosophila bifasciata*
Wolbachia of *Glossina brevipalpis*
0,95/76

Wolbachia of *Glossina morsitans centralis*
1/89

Wolbachia of *Glossina morsitans morsitans*
1/98

Wolbachia of *Glossina morsitans morsitans*
0,98/*

Wolbachia of *Glossina morsitans morsitans*
0,94/65

Wolbachia of *Ornithomya biloba* voucher rRNAob

1/100

H

Wolbachia of *Staphylinidae* sp.
1/100

Wolbachia of calyptrate fly
1/71

Wolbachia of *Suilia* sp.
0,76/*

Wolbachia of *Pheidole micula*
1/99

Wolbachia of *Glossina austeni*

0,99/54

Wolbachia of calyptrate fly
0,8/77

Wolbachia of *Glossina pallidipes*
1/100

Wolbachia of calyptrate fly

Wolbachia of *Zootermes angusticollis*

0,7/52

B

Wolbachia of *Tribolium confusum*
1/88

Wolbachia of *Teleogryllus taiwanemma*
1/88

Wolbachia of *Microlynchia galapogoensis* voucher MG6
0,96/85

Wolbachia of *Icosta* sp. 2 voucher P20

1/100

D

Wolbachia of *Glossina palpalis gambiensis*
Wolbachia of *Brugia malayi*
1/100

Wolbachia of *Wuchereria bancrofti*
1/100

Wolbachia of *Litomosoides sigmodontis*

C

Wolbachia of *Onchocerca ochengi*
1/100

Wolbachia of *Onchocerca volvulus*
1/100

Wolbachia of *Dirofilaria immitis*

0,96/64

F

Wolbachia of *Melophagus ovinus* voucher MO
Wolbachia of *Ocymyrmex picardi*
1/99

Wolbachia of *Opistophthalmus granifrons*
1/100

Wolbachia of *Opistophthalmus chaperi*
1/100

Wolbachia of *Opistophthalmus capensis*
1/96

Wolbachia of *Opistophthalmus litoralis*
1/99

Wolbachia of *Opistophthalmus latimanus*

1/90

Wolbachia of *Odontotermes horni*
0,92/*

Wolbachia of *Odontotermes horni*
1/100

Wolbachia of *Odontotermes* sp.

1/88

Wolbachia of *Apoica pallens*

0,92/74

0,54/*

Wolbachia of *Pseudolynchia* sp. voucher F5
1/94

Wolbachia of *Nycteribia kolenatii* voucher NK
0,96/63

Wolbachia of *Odontotermes horni*
0,55/*

Wolbachia of *Cimex lectularius*
1/90

Wolbachia of *Icosta* sp. 2 voucher L6
1/98

Wolbachia of *Icosta* sp. 1 voucher P8

1/63

1/90

E

1/100

Wolbachia of *Folsomia candida*

0.04