Hidden from plain sight: Novel *Chlamydiota* diversity emerging from screening genomic and metagenomic data.

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Abbreviations

MAG – Metagenome Assembled Genome

ANI – Average Nucleotide Identity

AAI – Average Amino-acid Identity

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Nucleotide sequence data

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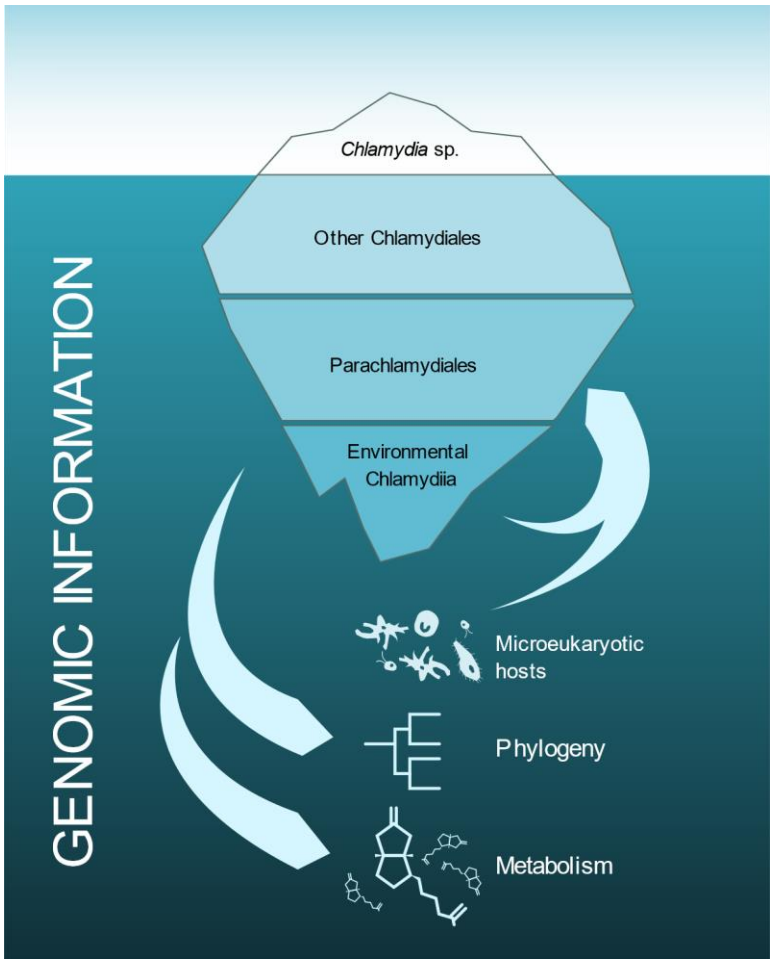
Abstract

Chlamydiota are an ancient and hyperdiverse Phylum of obligate intracellular bacteria. The best characterized representatives are pathogens or parasites of mammals, but it is thought that their most common hosts are microeukaryotes like Amoebozoa. The diversity in taxonomy, evolution, and function of non-pathogenic *Chlamydiota* are slowly being described. Here we use data mining techniques and genomic analysis to extend our current knowledge of *Chlamydiota* diversity and its hosts, in particular the Order *Parachlamydiales*. We extract one *Rhabdochlamydiaceae* and three *Simkaniaceae* genomes from NCBI Short Read Archive deposits of ciliate and algal genome sequencing projects. We then use these to identify a further 14 and 8 genomes respectively amongst existing, unidentified environmental assemblies. From these data we identify two novel clades with host associated data, for which we propose the names '*Candidatus* Sacchlamydia' (Family *Rhabdochlamydiaceae)* and '*Candidatus* Amphrikania' (Family *Simkaniaceae*), as well as a third new clade of environmental MAGs '*Candidatus* Acheromydia' (Family *Rhabdochlamydiaceae*). The extent of uncharacterized diversity within the *Rhabdochlamydiaceae* and *Simkaniaceae* is indicated by 16 of the 22 MAGs being evolutionarily distant from currently characterised genera. Within our limited data, we observe great predicted diversity in *Parachlamydiales* metabolism and evolution, including the potential for metabolic and defensive symbioses as well as pathogenicity. These data provide an imperative to link genomic diversity in metagenomics data to their associated eukaryotic host, and to develop onward understanding of the functional significance of symbiosis with this hyperdiverse clade.

Graphical Abstract



53 ## Introduction

54 *Chlamydiota* are a phylum of bacteria that are highly diverged from any other microbial clade,
55 such that they were for some time considered a separate kingdom of life (Prowazek, 1912;
56 Horn, 2008). Species within the group uniquely share a biphasic lifestyle, with an inert non-
57 dividing environmental stage and a reproductive intracellular stage (Kahane *et al.*, 1993;
58 Bastidas *et al.*, 2013; König *et al.*, 2017). These bacteria also share proteins that are associated
59 with chloroplast function in plants, which has been speculated to suggest a long-standing
60 relationship with cyanobacteria and algae (Brinkman *et al.*, 2002; Horn *et al.*, 2004). It has
61 also been proposed that ancient members of *Chlamydiota* facilitated the establishment of the
62 first photosynthetic proto-algal eukaryotes (Huang and Gogarten, 2007).

63 *Chlamydiota* are most well known as mammalian pathogens and were first described by the
64 ancient Chinese and Egyptians (Gruber, Lipozenčić and Kehler, 2015). However, the status of
65 the clade has been revised since the advent of DNA taxonomy and it is now recognised that
66 their host range is much broader. They are commonly found in free living amoeba species and
67 environmental samples, though here the original host is often not known (Horn, 2008).
68 Besides amoebae, they can also infect mammals, fish, worms, and a variety of arthropods
69 (Draghi *et al.*, 2004; Horn, 2008; Kjeldsen *et al.*, 2010; Halter *et al.*, 2022). There are potentially
70 many hundreds of unexplored families within *Chlamydiota* (Horn, 2008; Lagkouvardos *et al.*,
71 2014; König *et al.*, 2017). One order of *Chlamydiota*, the *Parachlamydiales*, are widely
72 observed and sequenced in environmental samples, but have little to no functional
73 information and recognised taxonomic affiliations are currently fluid (Horn, 2008; Nylund *et*
74 *al.*, 2018; Halter *et al.*, 2022).

75 Very recently, *Parachlamydia acanthamoebae* has been shown to protect its amoeba host
76 from giant viruses (Arthofer *et al.*, 2022). The mechanism for this defensive symbiosis is not
77 yet known, but *P. acanthamoebae* seems to prevent the virus from co-opting the amoeba
78 machinery, thus preventing the formation of viral factories. Additionally, *Neochlamydia* has
79 previously been implicated in defensive symbioses against *Legionella pneumophila* (Ishida *et*
80 *al.*, 2014; Maita *et al.*, 2018). These data indicate the potential these symbioses have as
81 hidden players in the biology, ecology, and evolution of microeukaryotes beyond a role as
82 pathogens. Moreover, this strong impact on host biology creates an imperative to investigate
83 symbioses in microeukaryotes outside of amoebae.

84 Sequence data provides an opportunity to estimate the level of uncharacterised diversity in
85 microbial symbionts. Two approaches are possible. First, genome sequencing projects for
86 eukaryotes commonly contain the reads from their associated symbionts within them.
87 Second, environmental sequencing projects allow the identification and assembly of draft
88 genomes without host association data. Here, we first screen the NCBI Sequence Read Archive
89 (SRA) databases for ciliates, macro- and micro-algae for the presence of *Chlamydiota* and
90 assemble 1 and 3 new draft genomes for the families, *Rhabdochlamydiaceae* and
91 *Simkaniaceae* respectively (both *Parachlamydiales*). We then use these genomes to identify
92 22 related MAGs from environmental metagenomic studies on online databases. Finally, we
93 conducted a phylogenetic and metabolic analyses of these, and existing genomes, to assess
94 their taxonomic placement and explore the potential impacts on their hosts.

95 # Materials and methods

96 ## Data collection

97 The presence of *Chlamydiota* bacteria was investigated across Algae and Ciliate SRA
98 databases on NCBI as described previously in Davison et al. (2022) using PhyloFlash to identify
99 deposits with evidence of Chlamydiota bacteria 16S rRNA sequence. Those SRA providing
100 evidence of *Chlamydiota* presence were taken forward to metagenomic binning and
101 assembly. We used GTDBtk to screen bins and found that all genomes recovered were related
102 to *Rhabdochlamydiaceae* or *Simkaniaceae*, so analyses from this point on were focused to
103 Parachlamydiales. Genomes assembled here were then used as baits to identify related
104 metagenomically assembled genomes (MAGs) in the NCBI nr database that were previously
105 not fully classified.

106 Existing *Parachalmydiales* genomes, as well as a *Clavichalmydia* and a *Chlamydia* genome
107 were downloaded from NCBI (see Supplementary data 1) alongside the GEM catalogue for
108 genomes identified by Köstlbacher et al. (Köstlbacher *et al.*, 2021; Nayfach *et al.*, 2021).

109 ## Metagenomic assembly and annotation

110 Minimap2, MEGAHIT and MetaBAT2 (Li *et al.*, 2015; Li, 2018; Kang *et al.*, 2019) were used to
111 assemble and bin metagenomic bacterial genomes from SRA data using the pipeline in
112 Davison (2022). Genome quality and identities were checked with CheckM and NCBI Blastn
113 (Camacho *et al.*, 2009; Parks *et al.*, 2015).

114 Four newly assembled genomes, 18 environmental MAGs, 82 previously established
115 *Parachlamydiaceae*, and 2 outgroup *Chlamydiaceae* were then passed to Anvio-7 to be

116 annotated with COG20 and KEGG kofams through their pangenomics pipeline (Aramaki *et al.*,
117 2020; Eren *et al.*, 2021; Galperin *et al.*, 2021; Davison, 2022).

118 ## Phlyogenomics and metabolic predictions

119 Clusters of protein homologs shared by 90% of the 106 genomes used were identified and
120 extracted through Anvio-7. We extracted 34 single copy core gene clusters that contain a total
121 of 3604 genes. A phylogeny partitioned by gene cluster was constructed with IQTREE, using
122 model finder plus, 1000 ultrafast bootstraps, and 1000 SH-alrt replicates. Models per partition
123 can be found in Supplementary data 2.

124 We used GTDBtk for taxonomic classification of the recovered genomes and to identify
125 taxonomic novelty among them. AAI was calculated for all genomes through kostalabs online
126 enveomics suite (Rodriguez-R and Konstantinidis, 2016). ANIb was calculated with pyANI
127 through anvio-7 (Supplementary data 1) (Pritchard *et al.*, 2016). The network was visualised
128 in Gephi 0.9 (Bastian, Heymann and Jacomy, 2009), with edges filtered for >0.6 AAI and >0.95
129 ANI scores. Annotations were added in Inkscape (Inkscape Project, 2020).

130 Anvio-7 using KEGG kofams was used to assess metabolic completeness for high quality
131 genomes exceeding a CheckM score of 90% completeness to minimize the loss of information
132 caused by incompleteness (Eisenhofer, Odriozola and Alberdi, 2023). Heatmaps were
133 constructed with Seaborn 0.12.1 in Python 3.10 and annotated with Inkscape (Rossum and
134 Drake, 2009; Waskom and Seaborn development team, 2020). Toxin-antitoxin systems were
135 identified with antiSMASH (Blin *et al.*, 2021). CrisprCAS finder (Couvin *et al.*, 2018) was used
136 to establish the presence of cas systems in all extracted genomes.

137 # Results

138 ## Genomes and Phylogeny

139 Four genomes were assembled from SRA data and an additional 22 recovered from the NCBI
140 GenBank non-redundant database (Table 1). Of these 22 genomes, 16 came from
141 environmental metagenome sequencing efforts that did not specifically target *Chlamydiota*,
142 and the six that were targeted came from a single paper. Two were Identified to species and
143 three to family level (Supplementary data 1). One of these, JAGXTH01, is a
144 *Rhabdochlamydiaceae* misidentified as a *Simkania* sp. (see Figure 1 and accession
145 GCA_018333475.1).

146 Several groups, like SM23-39 and most MCF types, are sufficiently different and diverse to
147 warrant their own family name. In literature, SM23-39 has been referred to as both '*Ca*.
148 Limichlamydiaceae' and '*Ca*. Anoxychlamydiaceae' (Pillonel, Bertelli and Greub, 2018;
149 Dharamshi *et al.*, 2020, 2023). Their classification seems to be mixed, with genomes identified
150 as SM23-39 also spanning a second proposed family '*Ca.* Enkichlamydiaceae' (Supplementary
151 data 1 and Köstlbacher *et al.*, (2021). Based on GTDBtk analysis we have opted for using the
152 clade name SM23-39 to refer to this clade to avoid naming confusion, and because all
153 genomes share family level classification by this method, as well as equivalent diversity with
154 *Rhabdochlamydiaceae* and *Simkaniaceae* (Figure 1 and 2). There is no set threshold for
155 classifying family or order level, and although GTDBtk does overclassify higher taxons
156 (Chaumeil *et al.*, 2020), it is currently the only objective way of designating family level
157 classification. Higher classifications can be added later as needed, but at this stage we do not
158 believe it is helpful to establish additional families or orders while the current within family
159 phylogeny is so fluid.

160 AAI and ANI results shown in Figure 2 support most existing genera and species clusters, but
161 also indicates the potential need for some genera to be split (e.g., *Simkania*) and some species
162 to be taxonomically combined ('*Ca*. Rhabdochlamydia oedothora' and '*Ca*. Rhabdochlamydia'
163 sp. W815 RHOW815).

164 Using AAI values with a cut off of 65%, we find objective support for naming the clade
165 containing MGLO01 as its own genus, '*Ca*. Arenachlamydia', as suggested in (Pillonel, Bertelli
166 and Greub, 2018). However, contrary to Pillonel et al. (2018), we do not find that they are
167 unique within *Chlamydiota* in lacking a Menaquinone biosynthesis/futalosine pathway
168 (Supplementary data 1). Amongst the genomes included in this study, all lack the futalosine
169 pathway (KEGG module M00930 and M00931) except for MCF-G and '*Ca.* Sorochlamydiaceae'
170 (clade CC-IV). The Menaquinone biosynthesis pathway (M00116) is partially present in all
171 except 13 genomes, of which span several different clades across *Parachlamydiales*
172 (Supplementary data 1). Other clades form putative genera groups of four or more genomes
173 that are simply unnamed. *Simkaniaceae* and *Rhabdochlamydiaceae* in particular lack solid
174 categorisation, each having only two previously named genera (Figure 1 and 2). Further, the
175 genomic diversity displayed within both families is extensive. Some single genomes possess
176 more than ten times more unique genes than genes shared across all genomes (Figure 3),
177 though this could be an artifact of divergent orthologs not clustering together. It is very likely
178 that *Parachlamydiales* will need to be split into multiple orders as more data becomes
179 available, and revision of family level affiliation, to better describe this ancient clade of
180 bacteria.

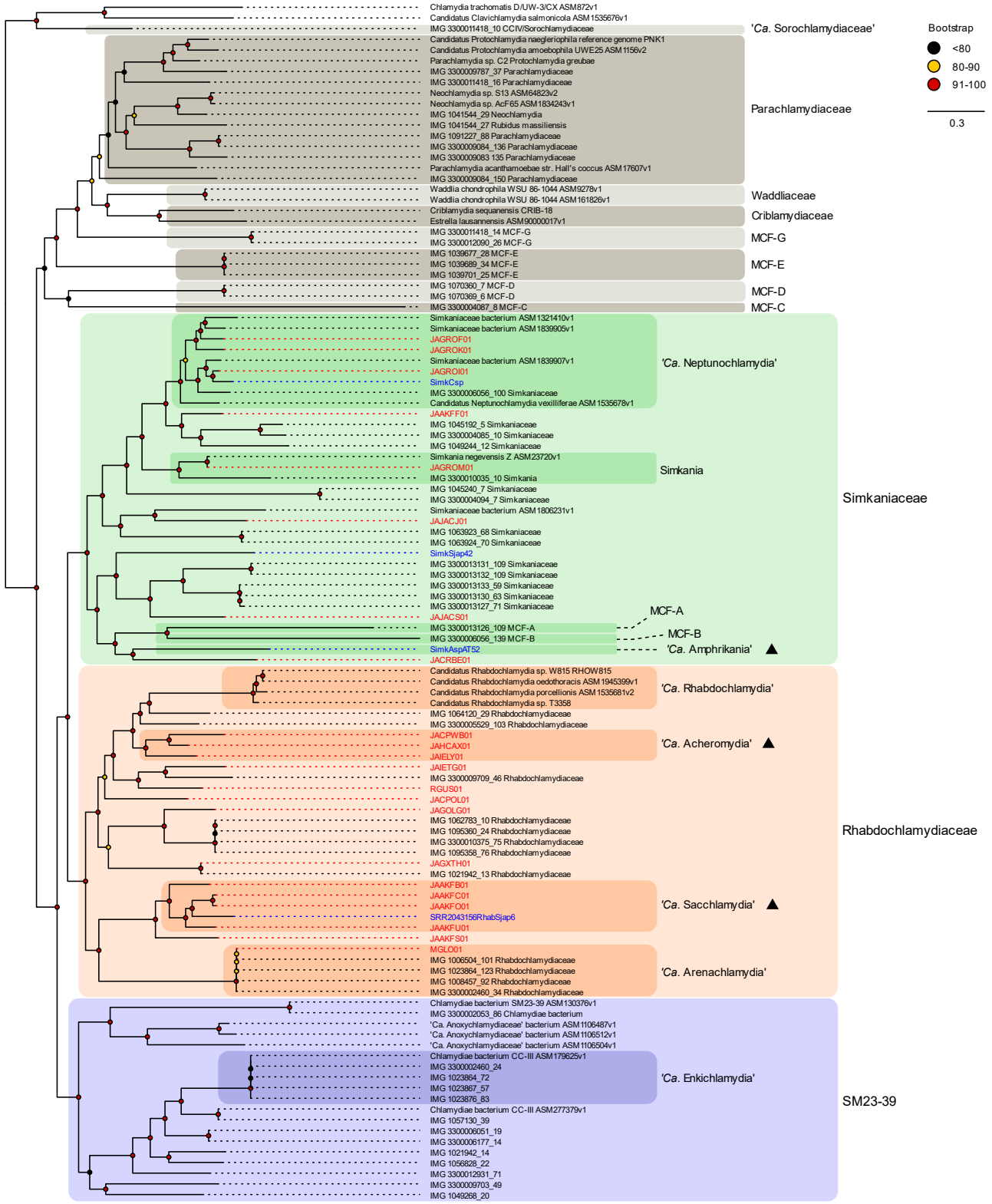


Figure 1. Genome wide phylogeny of *Parachlamydiales*. Maximum likelihood (ML) phylogeny of *Parachlamydiales* constructed from 34 single copy gene clusters that contain a total of 3604 genes. New genomes are indicated by ▲ with blue labels for genomes derived from host SRA bins, and red for MAGS without host assocition data. Bootstrap values based on 1000 replicates are indicated with coloured circles (red = 91-100, yellow = 81-90, black <= 80).

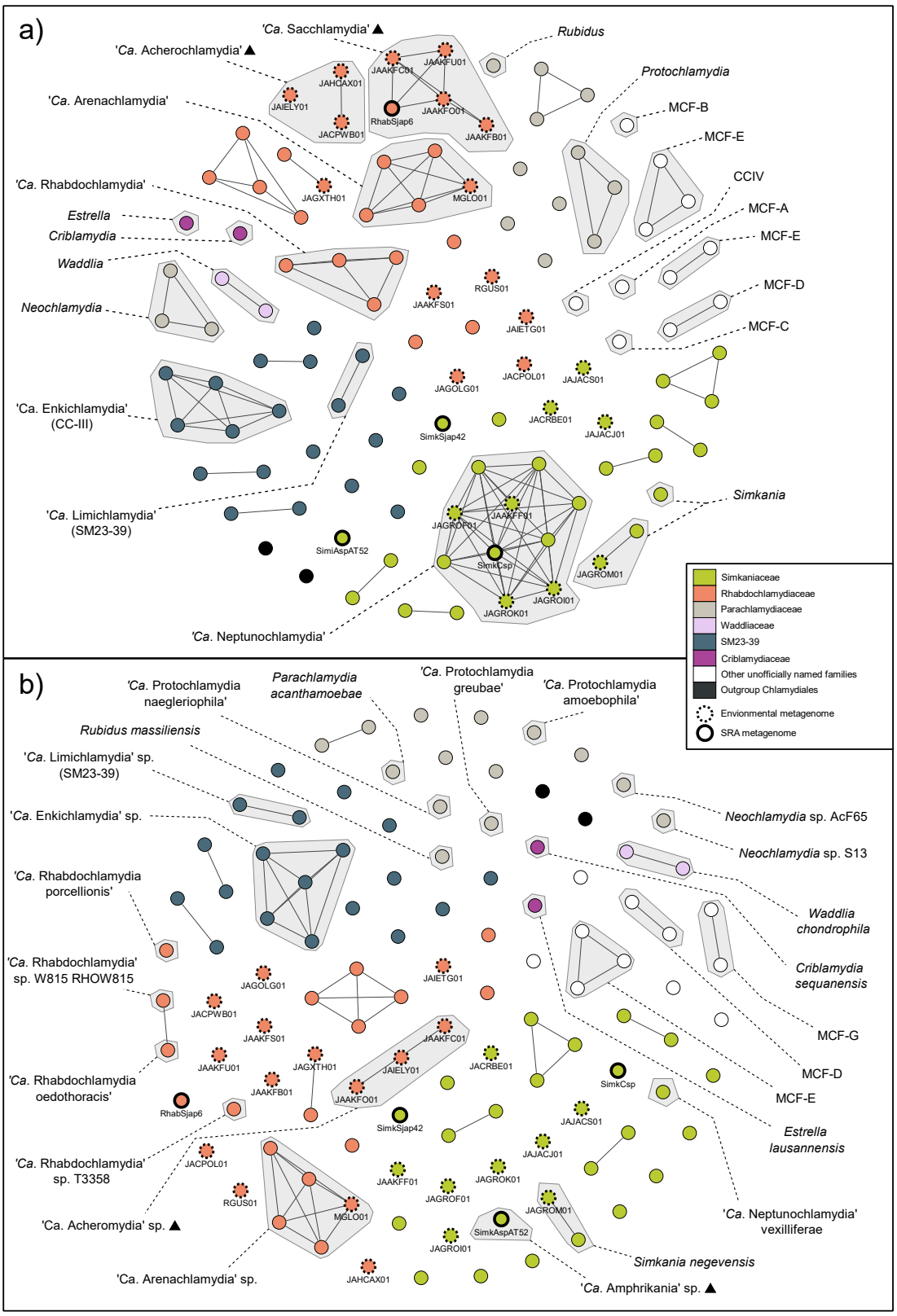


Figure 2. Genus and species level clustering across *Parachlamydiales*. Frutcherman Reingold networks of pairwise a) Average Amino Acid Identity (AAI) with edge weights >65% similarity and b) Average Nucleotide Identity (ANI) with edge weights >95% similarity across all genomes. AAI and ANI illustrate genus and species boundaries, respectively. Proposed genus names are indicated with a ▲.

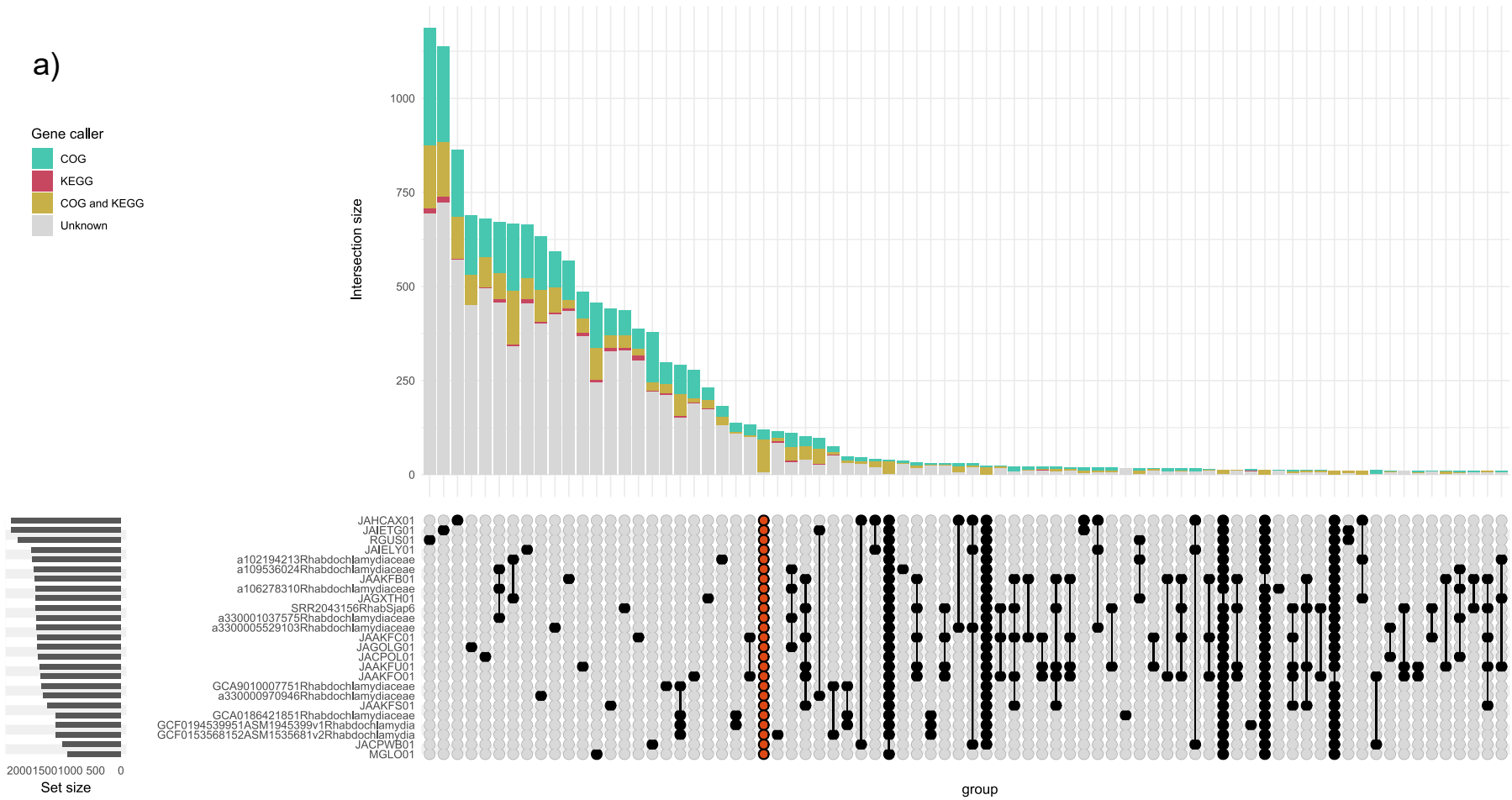


Figure 2a. Gene content comparison for *Rhabdochlamydiaceae* and *Simkaniaceae*. An upsetplot illustrating the disparity of gene presence absence across the families a) *Rhabdochlamydiaceae* and b) *Simkaniaceae*. Vertical bars illustrate the number of genes present shared across compared genomes, indicated by black circles beneath each bar. Orange circles indicate the core genome shared by all genomes in the comparison.

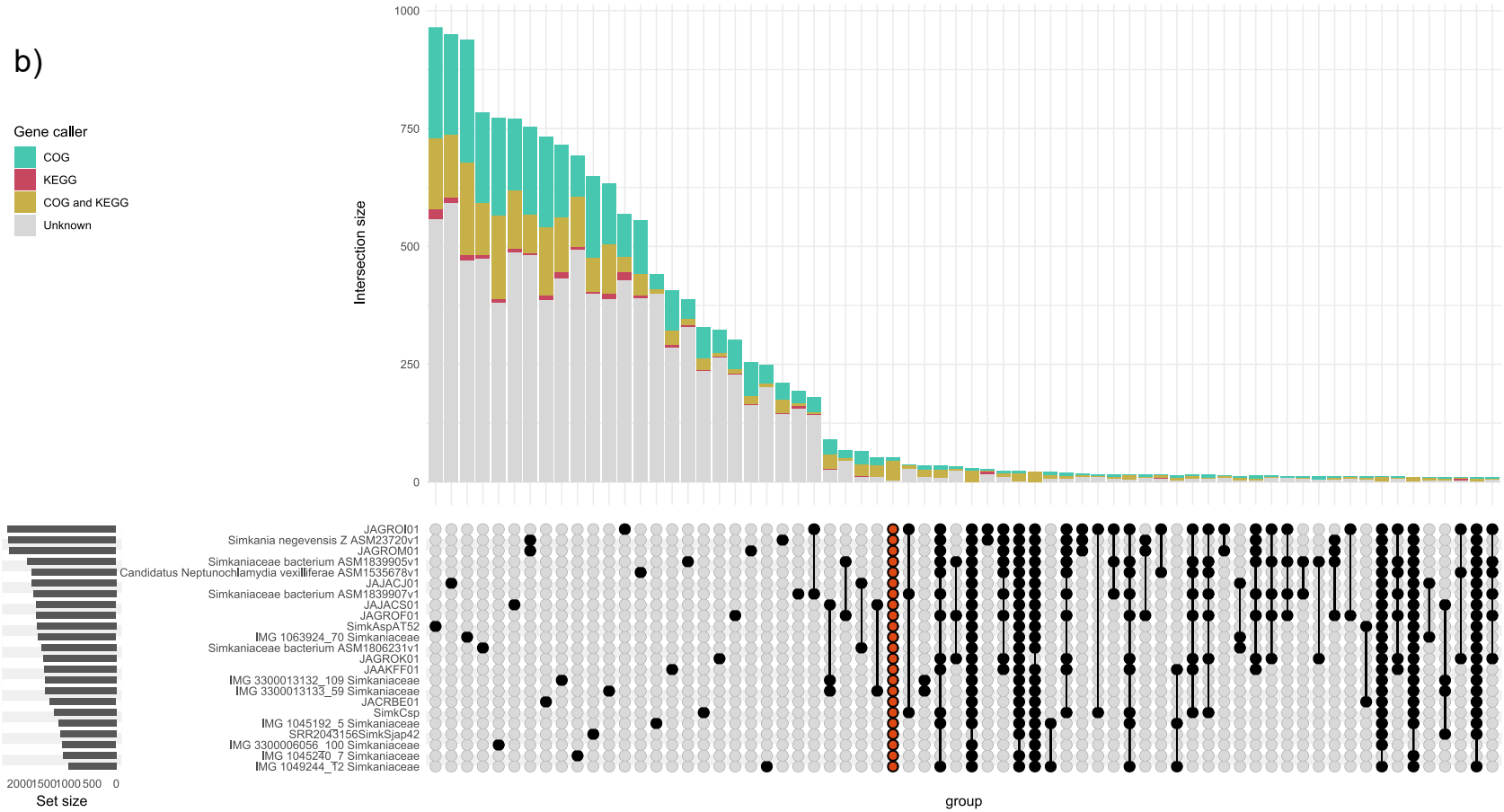


Figure 3b. Gene content comparison for *Rhabdochlamydiaceae* and *Simkaniaceae*. An upset plot illustrating the disparity of gene presence absence across the families a) *Rhabdochlamydiaceae* and b) *Simkaniaceae*. Vertical bars illustrate the number of genes present shared across compared genomes, indicated by black circles beneath each bar. Orange circles indicate the core genome shared by all genomes in the comparison.

203 ## Proposed Taxonomy

204 Based on AAI, ANI, GTDBtk analysis, metabolic results, and phylogeny, we find evidence for at
205 least three new genus-level clusters within Rhabdochlamydiaceae and Simkaniaceae. The
206 genera we propose contain either multiple high-quality environmental genomes found with
207 the methods described in this study, or are high-quality assemblies extracted from potential
208 host SRAs.

209 '*Candidatus* Sacchlamydia' gen. nov. - For the cluster that contains RhabSjap6, a host
210 associated genome, named after the putative host *Saccharina japonica* and the pattern of
211 using "-chlamydia" as the suffix for Chlamydiota genera.

212 '*Candidatus* Amphrikania' gen. nov. - A second new clade with host association data lies
213 within the Simkaniaceae (the genome named SimkAspAT52). Amphri- after Amphritrite
214 Queen of the Sea and echoing its marine host *Amoebophyra*, and -kania after the type genus
215 for the family Simkaniaceae, *Simkania*.

216 Similarly to SimkAspAT52, clade SimkSjap42 is likely a new species group but will not be
217 named because it has genome that is only 65% complete in CheckM analysis (Figure 1., Table
218 1).

219 '*Candidatus* Acheromydia' gen. nov. - For the clade including the MAGs JAHCAX01 and
220 JACPWB01 which share >65% AAI similarity. For convenience, and so as not to over inflate
221 this group with new names, I also include the relatively closely related genome JAIEL01 (>62%
222 AAI similarity). This genus is named after Acheron (Acher-) one of five Greek rivers of the
223 underworld that occasionally surfaces above ground. The MAGs come from drinking water,
224 activated sludge and ground water. -mydia from *Chlamydia*.

225 ## Metabolism

226 Parachlamydiales have a broad array of metabolic pathways (Figure 4 and Supplementary
227 data 1), including nutritional and defensive systems. Of note, several have mostly complete B
228 vitamin synthesis pathways (biotin, riboflavin and thiamine), known to be associated with
229 beneficial contributions to the host in other symbioses with intracellular bacteria.

230 Some Parachlamydiales have uroporphyrinogen decarboxylase and uroporphyrinogen-III
231 synthase which form part of several biosynthesis pathways including heme, chlorophyll,
232 cobalamin, and siroheme. In addition, some have incomplete phylloquinone (vitamin K1)
233 biosynthesis pathways. All Parachlamydiales genomes here also have complete Type III
234 secretion systems and incomplete gene sets associated with flagellar assembly
235 (Supplementary data 1). Several terpenoid synthesis pathways are also present, including
236 dTDP-L-rhamnose biosynthesis.

237 We find that KEGG Kofam identifies CRISPR-associated protein Cas2 in all *Parachlamydiales*
238 studied in this study, including *P. acanthamoeba*. Several have additional types of CRISPR/Cas
239 associated genes (Supplementary data 1). CRISPRcas-finder identifies cas and cas spacers in
240 the four MAGs extracted from the SRA samples (Supplementary data 1) and only RhabSjap6
241 appears to possess both CRISPR elements and a cas cluster. Whether these genes are
242 functional or associated with defensive symbiosis against phages based purely on metabolic
243 prediction is unknown; the class of cas is not predictive of their function.

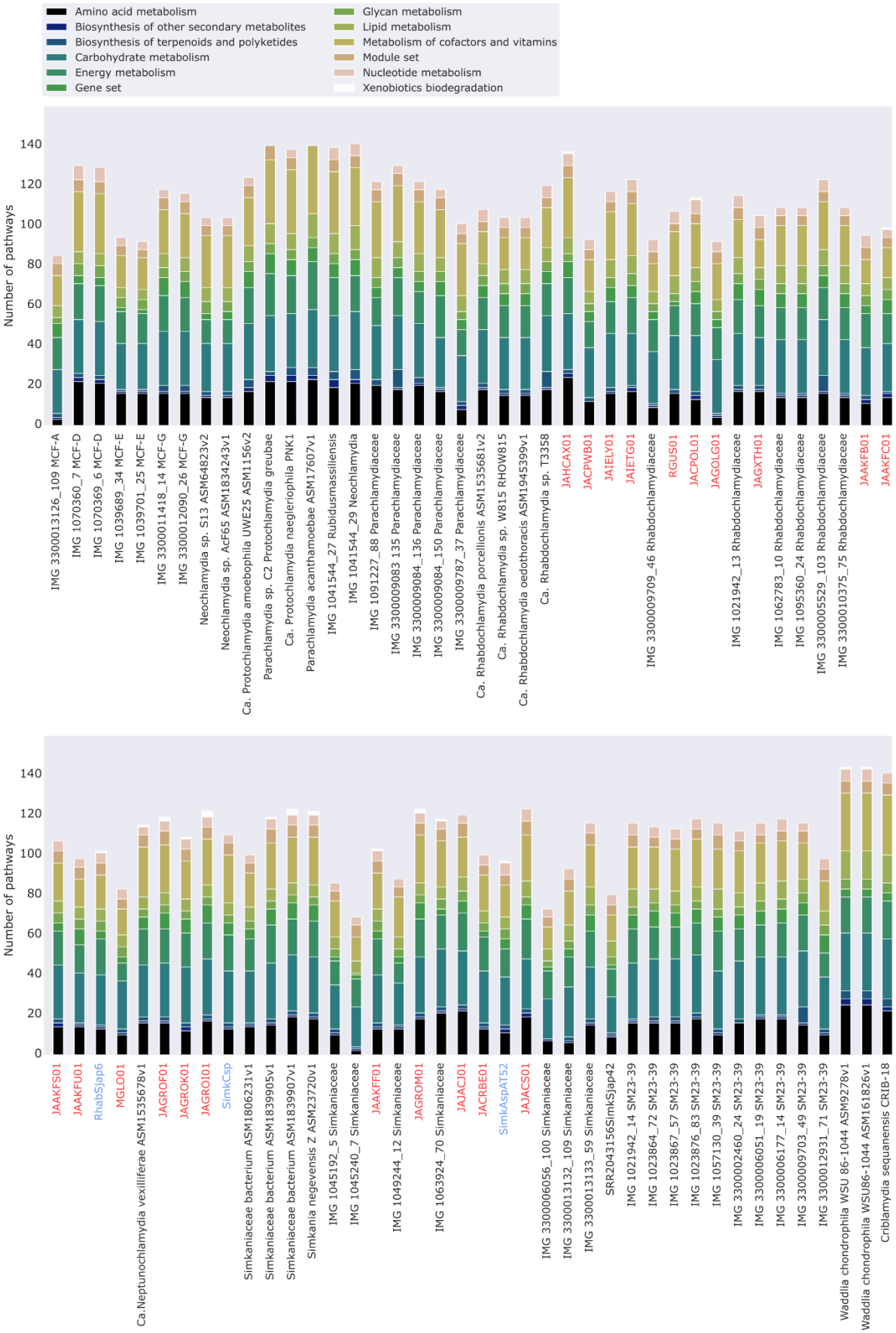


Figure 3. The number of each type of metabolic pathway found in each *Chlamydiota* genome. Red indicates environmental MAGs; blue indicates MAGs assembled in this study.

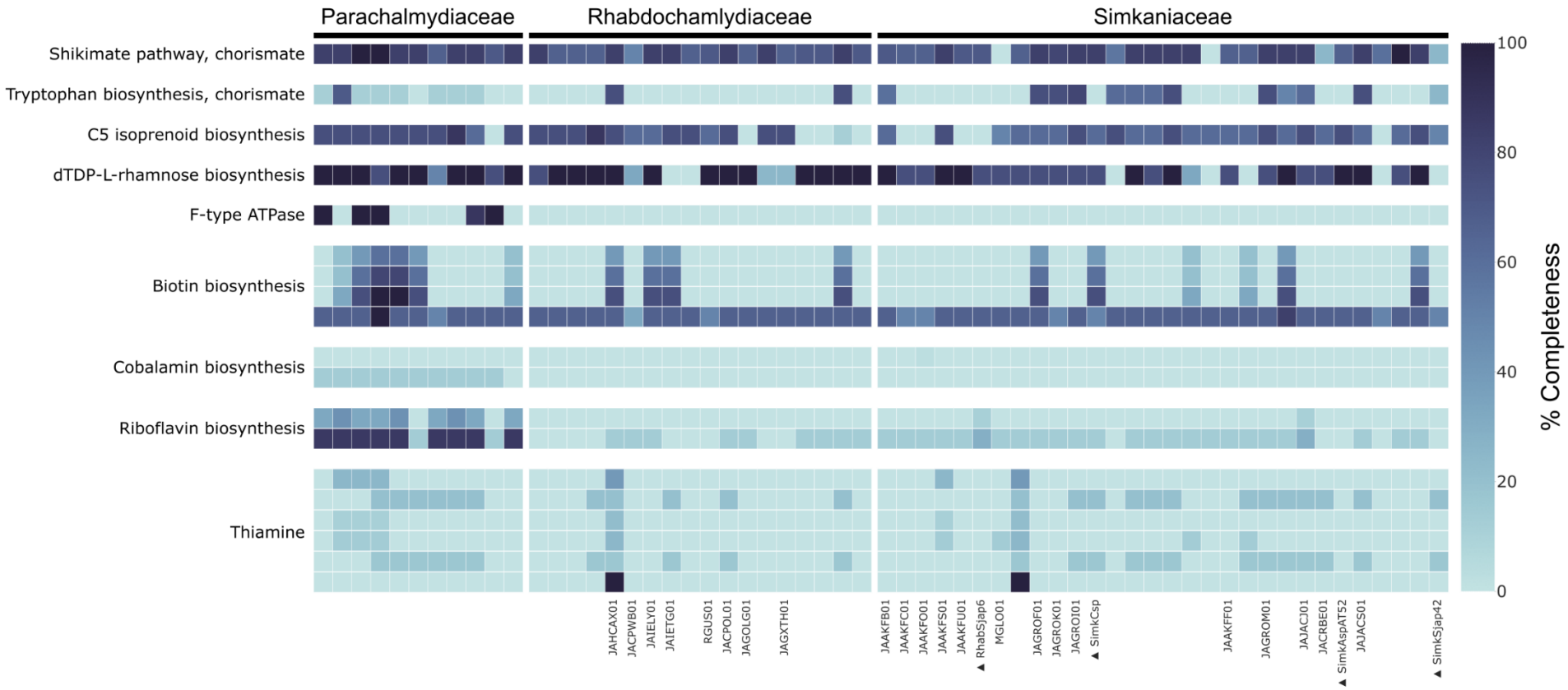


Figure 4. Heatmap of the completeness of KEGG metabolic pathways of interest across the *Parachlamydiaceae, Rhabdochlamydiaceae and Simkaniaceae*. Full metabolic pathway completeness for all genomes and all other pathways are available in Supplementary data 1. New genomes assembled in this study are indicated by ▲.

251 # Discussion

252 ## Genomes and Phylogeny

253 We observe high genomic, phylogenetic, and metabolic diversity in the currently named
254 families, genera, and within species of *Chlamydiota*, in line with other studies (Horn, 2008;
255 Lagkouvardos *et al.*, 2014; König *et al.*, 2017). The current taxonomy is not sufficient and has
256 been left undefined to the detriment of these bacteria, in part due to the nature of the clade
257 being so broad and data being so sparse. This has resulted in some genomes such as *Rubidus*
258 *massiliensis* and JAGXTH01 being miscategorised on NCBI. *Rubidus massiliensis* is categorised
259 as *Chlamydiaceae* rather than *Parachlamydiaceae,* as proposed in the study where it was first
260 described (Bou Khalil *et al.*, 2016) and as shown in Figure 1. MAG JAGXTH01 is identified as a
261 *Simkania* sp., whereas we find it falls within the *Rhabdochlamydiaceae*.

262 Prior to this study, only two Environmental MAGs found had been assigned a taxonomic
263 classification lower than family, and one of those two is misidentified (Supplementary data
264 1). At this stage we do not believe it is helpful to split existing family clades like SM23-39 into
265 multiple families, especially since they are supported by GTDBtk analysis. There is also some
266 confusion in literature for the taxonomy for some clades: '*Ca*. Limichlamydiaceae' (Pillonel,
267 Bertelli and Greub, 2018) is included in '*Ca*. Anoxychlamydiaceae' (Dharamshi *et al.*, 2023),
268 and '*Ca*. Enkichlamydiaceae' (Pillonel, Bertelli and Greub, 2018) == Chlamydiae clade III
269 (Dharamshi *et al.*, 2023).

270 We believe the splitting of *Parachlamydiales* into three new orders are in some respects
271 warranted: 'C*a*. Anoxychlamydiales', 'C*a*. Simkaniales' (which includes *Rhabdochlamydiaceae*
272 and *Simkaniaceae*) and 'C*a*. Amoebachlamydiales' (for *Parachlamydiaceae*) (Dharamshi *et*

273 *al.*, 2023). We could also argue that *Rhabdochlamydiaceae* and *Simkaniaceae* will eventually
274 need their own Order classifications based on the high gene content diversity within the
275 families (figures 3a and 3b). However, we do not believe it is helpful to split *Parachlamydiales*
276 into further orders at this time while most families and genera have not been properly
277 defined. Additionally, Order classification has no robust criteria and the erection of further
278 Orders may contribute to confusion around the taxonomy of *Chlamydiota*.

279 Metabolism

280 Vitamin B biosynthesis is known to be associated with beneficial contributions to the host in
281 other symbioses with intracellular bacteria. However, all characterised Parachlamydiales have
282 biphasic lifestyles, and many are known pathogens, so the presence of B vitamin modules
283 does not necessarily indicate potential benefit to the host. For instance, pathogenic
284 *Chlamydia* with bioY genes have previously been observed to uptake biotin from host cells
285 (Fisher *et al.*, 2012).

286 If Parachlamydiales do form beneficial symbioses with their host it is feasibly associated with
287 photosynthesis through vitamin Phylloquinone synthesis or chlorophyll synthesis.
288 Phylloquinone is produced and used by plants, algae, and cyanobacteria in photosynthesis.
289 Most *Parachlamydiales* genomes also have a complete chorismate synthase pathway (Figure
290 5, Supplementary data 1), an important intermediate product for phylloquinone production,
291 as well as intermediates for alkaloids and salicylic acid that are important in plant defence
292 systems (Hamberger *et al.*, 2006; Shanmugabalaji *et al.*, 2022). It should be noted that
293 chorismate can also be used to synthesize a variety of other vital aromatic compounds such
294 as ubiquinone, which is common in bacteria (Dosselaere and Vanderleyden, 2001).

295 There is also a possibility that these bacteria form defensive symbioses, as seen in the case of
296 viral protection of amoebae by *P. acanthamoeba* (Arthofer *et al.*, 2022). Unfortunately, we
297 do not know how *P. acanthamoeba* blocks viral factory formation, so we cannot identify any
298 associated pathways through homology. The presence of CRISPR/cas systems in some
299 genomes could indicate a potential mechanism for this trait, but we do not know that they
300 are functional, and, if they are, they may be associated with the free living stage rather than
301 antiviral activity in the host. CRISPR/Cas Phage defence are common across bacteria but
302 notably rare in microbial symbionts (Deveau, Garneau and Moineau, 2010). They have also
303 previously been found in *Protochlamydia* genomes and other *Chlamydiota* (Deveau, Garneau
304 and Moineau, 2010; Bertelli *et al.*, 2016; Köstlbacher *et al.*, 2021).

305 An alternative route for defensive symbiosis could be terpenoid production. Terpenoids such
306 as dTDP-L-rhamnose are associated with plant-bacteria symbioses as well as pathogenicity
307 (Ma, Pan and McNeil, 2002; Jofré, Lagares and Mori, 2004; French, 2017; Jiang *et al.*, 2021).
308 Terpenoids are also used by algae themselves in defence against threats such as bacteria and
309 heavy metals (French, 2017; Karimi *et al.*, 2019). Some Red algae use bacteria-like pathways
310 to produce their terpenoids (Wei *et al.*, 2019).

311 Conclusions

312 Chlamydiota are hyper diverse and under described. We have added to the known
313 phylogenies of the *Parachlamydiales* families: *Simkaniaceae* and *Rhabdochlamydiaceae*. In
314 addition, we clarify the status of two additional *Rhabdochlamydiaceae* and one *Simkaniaceae*
315 genera, for which we propose the names '*Ca*. Sacchlamydia', '*Ca.* Acherochlamydia', and '*Ca.*
316 Amphrikania'. The metabolic potential of *Chlamydiota* is likewise highly diverse, with several
317 clusters of genes that could be associated with both defensive and nutritional symbiosis or

318 otherwise associated with pathogenicity. Closer study of the metabolic interactions between

319 *Parachlamydiales* and their microeukaryotic hosts are required to elucidate if and how they

320 affect each other.

321 # Tables

322 Table 1. Select genome metadata for newly assembled Rhabdochlamydiaceae and Simkaniaceae genomes and previously assembled NCBI
323 environmental MAGs. Full metadata can be found in Supplementary data 1.

Name	Bacteria accession	Isolate Name	Family	Source accession	Host Type	Source	CheckM completion score	CheckM contamination	Genome size	Number of contigs	GC content %	Completion status
Genomes assembled in this study												
SimiAspAT52	SAMN32091777	-	*Simkaniaceae*	SRR3080743	Algae	*Amoebophrya* sp. AT5.2	91.55	0	1,800,129	71	36.81	contig
SimkCsp1	SAMN32091778	-	*Simkaniaceae*	SRR9841583	Ciliate	*Chilodochona* sp.	90.65	0.23	1,516,029	217	39.52	contig
RhabSjap6	SAMN32091779	-	*Rhabdochlamydiaceae*	SRR2043156	Algae	*Saccharina japonica*	91.05	0.9	1,734,283	4	45.53	contig
SimkSjap42	SAMN32091780	-	*Simkaniaceae*	SRR2043156	Algae	*Saccharina japonica*	65.79	1.73	972,015	316	43.90	contig
Existing environmental MAGs												
JAAKFB01	GCA_011065235.1	*K1000_chlam_2*	*Rhabdochlamydiaceae*	PRJNA504765	Unknown	marine sediment	82.56	2.73	1,738,011	252	44.0	contig
JAAKFC01	GCA_011065225.1	*K1000_chlam_3*	*Rhabdochlamydiaceae*	PRJNA504765	Unknown	marine sediment	78.01	2.03	1,662,393	270	41.5	contig
JAAKFF01	GCA_011065165.1	*K1060_chlam_2*	*Simkaniaceae*	PRJNA504765	Unknown	marine sediment	90.2	0	1,625,346	143	46.5	contig
JAAKFO01	GCA_011064755.1	*K940_chlam_6*	*Rhabdochlamydiaceae*	PRJNA504765	Unknown	marine sediment	82.02	3.6	1,505,120	432	42.5	contig
JAAKFS01	GCA_011064965.1	*KR126_chlam_1*	*Rhabdochlamydiaceae*	PRJNA504765	Unknown	marine sediment	92.91	1.35	1,614,069	25	46.5	contig
JAAKFU01	GCA_011064915.1	*KR126_chlam_3*	*Rhabdochlamydiaceae*	PRJNA504765	Unknown	marine sediment	84.12	2.25	1,677,468	185	42.5	contig
JACPOL01	GCA_016185065.1	*NC_groundwater_600_Pr5_B-0.1um_49_25*	*Rhabdochlamydiaceae*	PRJNA640378	Unknown	groundwater	95.61	0.68	1,865,878	10	48.5	contig

JACPWB01	GCA_016197265.1	*NC_groundwater_800_Pr6_B-0.1um_47_6*	*Rhabdochlamydiaceae*	PRJNA640378	Unknown	groundwater	76.96	5.69	1,071,557	362	46.5	scaffold
JACRBE01	GCA_016213235.1	*NC_groundwater_1612_Pr3_B-0.1um_36_11*	*Simkaniaceae*	PRJNA640378	Unknown	groundwater	82.09	1.04	1,468,519	59	36.0	contig
JAGOLG01	GCA_017994195.1	*Gw_Bodden_bin_68*	*Rhabdochlamydiaceae*	PRJNA524094	Unknown	wastewater	89.02	1.49	1,679,597	130	42.5	contig
JAGROF01	GCA_020440405.1	*HKST-UBA11*	*Simkaniaceae*	PRJNA432264	Unknown	Saline, activated sludge	80.23	0.34	1,731,737	299	44.5	scaffold
JAGROI01	GCA_020440305.1	*HKST-UBA14*	*Simkaniaceae*	PRJNA432264	Unknown	saline, activated sludge	96.28	0.56	2,560,732	197	39.0	scaffold
JAGROK01	GCA_020440285.1	*HKST-UBA16*	*Simkaniaceae*	PRJNA432264	Unknown	Saline, activated sludge	88.85	1.52	1,696,508	180	42.5	contig
JAGROM01	GCA_020440265.1	*HKST-UBA18*	*Simkaniaceae*	PRJNA432264	Unknown	Saline, activated sludge	92.44	5.63	2,391,591	302	41.5	Scaffold
JAGXTH01	GCA_018333475.1	*CROMO_BS_87_3_0*	*Rhabdochlamydiaceae*	PRJNA672823	Unknown	groundwater	84.46	4.05	1,945,191	100	42.0	Contig
JAHCAX01	GCA_019634675.1	*ACE_VER1*	*Rhabdochlamydiaceae*	PRJNA725625	Unknown	activated sludge	96.96	0.68	2,665,416	21	46.5	Contig
JAIELY01	GCA_019752555.1	*co.spades.CONCOCT.1kb_142*	*Rhabdochlamydiaceae*	PRJNA745370	Unknown	drinking water	95.61	0.68	2,067,720	42	44.0	contig
JAIETG01	GCA_019745395.1	*time.spades.CONCOCT.1kb_023*	*Rhabdochlamydiaceae*	PRJNA745370	Unknown	drinking water	93.69	1.35	2,621,400	37	39.5	Contig
JAJACJ01	GCA_022601575.1	*O_S4.1_1*	*Simkaniaceae*	PRJNA742377	Unknown	sponge, *Haliclona oculata*	95.95	0	1,780,284	3	45.0	Scaffold
JAJACS01	GCA_022601395.1	*O_S4.2_37*	*Simkaniaceae*	PRJNA742377	Unknown	sponge, *Haliclona oculata*	94.93	0.68	1,870,253	48	38.5	contig
MGLO01	GCA_001796065.1	-	*Rhabdochlamydiaceae*	PRJNA288027	Unknown	groundwater	84.46	0.17	1,177,368	32	49.0	Scaffold
RGUS01	GCA_010027655.1	*WB9_2_327*	*Rhabdochlamydiaceae*	PRJNA495371	Unknown	Freshwater	91.44	13.91	2,063,700	445	38.0	contig

Supplementary material captions

Supplementary data 1 – metadata and metabolic data for genomes used in this study.

Supplementary data 2 – tree partition models for Figure 1.

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472

473 ## Figure captions

474 **Figure 1. Genome wide phylogeny of Parachlamydiales.** Maximum likelihood (ML) phylogeny
475 of Parachlamydiales constructed from 34 single copy gene clusters that contain a total of 3604
476 genes. New genomes are indicated by ▲ with blue labels for genomes derived from host SRA
477 bins, and red for MAGS without host assocition data. Bootstrap values based on 1000
478 replicates are indicated with coloured circles (red = 91-100, yellow = 81-90, black <= 80).

479 **Figure 2. Genus and species level clustering across *Parachlamydiales*.** Frutcherman Reingold
480 networks of pairwise a) Average Amino Acid Identity (AAI) with edge weights >65% similarity
481 and b) Average Nucleotide Identity (ANI) with edge weights >95% similarity across all
482 genomes. AAI and ANI illustrate genus and species boundaries, respectively. Proposed genus
483 names are indicated with a ▲.

484 **Figure 3a. Gene content comparison for *Rhabdochlamydiaceae* and *Simkaniaceae*.** An
485 upsetplot illustrating the disparity of gene presence absence across the families a)
486 *Rhabdochlamydiaceae* and b) *Simkaniaceae*. Vertical bars illustrate the number of genes
487 present shared across compared genomes, indicated by black circles beneath each bar.
488 Orange circles indicate the core genome shared by all genomes in the comparison.

489 **Figure 3b. Gene content comparison for *Rhabdochlamydiaceae* and *Simkaniaceae*.** An upset
490 plot illustrating the disparity of gene presence absence across the families a)
491 *Rhabdochlamydiaceae* and b) *Simkaniaceae*. Vertical bars illustrate the number of genes
492 present shared across compared genomes, indicated by black circles beneath each bar.
493 Orange circles indicate the core genome shared by all genomes in the comparison.

494 **Figure 4. The number of each type of metabolic pathway found in each *Chlamydiota***
495 **genome.** Red indicates environmental MAGs; blue indicates MAGs assembled in this study.

496 **Figure 5. Heatmap of the completeness of KEGG metabolic pathways of interest across the**
497 ***Parachlamydiaceae*, *Rhabdochlamydiaceae* and *Simkaniaceae*.** Full metabolic pathway
498 completeness for all genomes and all other pathways are available in Supplementary data 1.
499 New genomes assembled in this study are indicated by ▲.