Variation and diversification of the microbiome of *Schlechtendalia chinensis* on two alternate host plants

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Abstract

Schlechtendalia chinensis, a gall-inducing aphid, has two host plants in its life cycle. Its wintering host is a moss (typically *Plagiomnium maximoviczii*) and its main host is *Rhus chinensis* (Sumac), on which it forms galls during the summer.. This study investigated bacteria associated with *S. chinensis* living on the two different host plants by sequencing 16S rRNAs. A total of 183 Operational Taxonomic Units (OTUs) from 50 genera were identified from aphids living on moss, whereas 182 OTUs from 49 genera were found from aphids living in Sumac galls. The most abundant bacterial genus among identified OTUs from aphids feeding on both hosts was *Buchnera*. Despite similar numbers of OTUs, the composition of bacterial taxa showed significant differences between aphids living on moss and those living on *R. chinensis*. Specifically, there were 12 OTUs from 5 genera (family) unique to aphids living on moss, and 11 OTUs from 4 genera (family) unique to aphids feeding in galls on *R. Chinensis*. Principal Coordinate Analysis (PCoA) also revealed that bacteria from moss-residing aphids clustered differently from aphids collected from galls. Our results provid a foundation for future analyses on the roles of symbiotic bacteria in plant - aphid interactions in general, and how gall-specific symbionts differ in this respect.

Introduction

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41 Insects harbour a wide range of symbiotic microbes, but the actual species

42 composition can vary between different developmental stages [1, 2]. In general,

43 bacterial symbionts are comprised of two kinds: obligate symbionts and facultative

44 symbionts. Obligate endosymbionts are necessary for insect growth and development,

45 and usually supply important nutrients such as essential amino acids to the host insect

46 [3, 4]. In contrast, facultative symbionts are not essential for growth and development

47 of their host, but may improve fitness. For instance, some facultative symbionts boost

48 the defense system of the host and thus enhance resistance to natural enemies and

49 pathogens [5-7]. Typically, facultative symbionts have not shared a long evolutionary

50 relationship with their host insects [8].

51 Aphids are a large group of insects belonging to the Aphidiodea superfamily.

52 Many aphid species are destructive pests of crops and forests. Symbiosis with

53 microorganisms is critical for the development of aphids. One of the essential

54 symbiotic bacteria associated with aphids is *Buchnera aphidicola*, an obligate

55 endosymbiont that provides essential amino acids, which are either absent or scarce in

56 plant sap [9]. In addition to its obligate symbionts, aphids can also harbor several

57 facultative bacterial symbionts, which can be mutualistic in the context of various

58 ecological interactions. *Serratia symbiotica* is one of the most common facultative

59 symbiotic bacteria in aphids [10]. S. *symbiotica*, together with other symbionts,

60 provides aphids with protection against parasitoids [11]. Because of different

61 ecological habitats, different aphid species harbor specific bacterial taxa for specific

62   functions [12].

63   The aphid *Schlechtendalia chinensis*, a member of the subfamily Pemphiginae, is

64   a beneficial species with great economic and medicinal values [13, 14]. The aphid

65   induces the formation of large, single-chambered galls on its host plant, and some

66   chemicals of the galls are used as ingredientsin traditional Chinese medicine to treat

67   various conditionsincluding cough, diarrhea, night sweats, dysentery, and internal

68   bleeding [15]. In the mining industry, some applications use chemicals from *S.*

69   *chinensis*-induced galls to extract rare metals. In addition to its economic and

70   medicinal values, *S. chinensis* also has a fascinating biology. The aphid has a life

71   cycle of sexual and asexual reproduction, and a switch of host plant species during its

72   life cycle. The primary host of *S. chinensis* is *Rhus chinensis* (Sumac tree) and the

73   secondary host is *Plagiomnium maximoviczii* (moss). In early spring in China,

74   sexuparae migrate from mosses to the trunk of Sumac trees to produce sexually

75   reproductive females and males that subsequently mate to produce fundatrix, which

76   feed on rachide wings and induce the formation of galls [16, 17]. Within a gall, *S.*

77   *chinensis* undergoes cyclical parthenogenesis for multiple generations to increase

78   population sizes rapidly. In late fall, the galls break up and the alate fundatrigeniae of

79   *S. chinensis* emerge fromgalls and relocateto their secondary host mosses, where

80   aphids over-winter.

81   Based on its unique biology and its ability to induce galls, we hypothesized that

82   *S. chinensis* harbors a set ofunique symbiontic taxa, thatare likely to play critical roles

83   in its biology, such as mediating adaptive responses on different hosts, or contributing

84   toits gall-inducing ability. The rapid advance in high throughput sequencing

85   technologies has made it possible to comprehensively investigate the compositions of

86   microbial community in small insects [18]. In this study, we examined the diversity of

87   bacterial communities associated with *S. chinensis* in different hosts via next-

88   generation sequencing. Specifically, we sequenced the variable regions 4-5 of 16S

89   rRNA amplicons using the Illumina sequencing platform (using barcoded Illumina

90   paired-end sequencing, BIPES) to characterize the composition and diversity of

91   microbiomes during different developmental stages of *S. chinensis.*

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Materials and methods

Aphid collection

We collected aphid samples corresponding to nine time points (one per month) directly from host plants from March to November, 2015, in Kunming, Yunnan province, China. Each condition was represented by three independent replicates. In order to observe potential dynamic changes in the diversity of microbal taxa, samples were collected every month, and for consistency, we chose the middle of each month. The April samples represented the fundratrix stage.. Samples from March, April, and November were taken as the group of insects from the host moss, whereas the remaining samples were taken from Sumac trees.

To obtain insects from galls, individual galls were soaked in 75% ethanol by gently shaking for 30 s. The gall was washed three times with doubly distilled (dd) water. Using sterile conditions, an incision into the gall was carried outwith a blade. Aphids were taken out individually with forceps and transferred into individual tubes. After removing any environmental contaminants, the insect samples were frozen at −80 °C for subsequent genomic DNA extraction. For moss-residing aphids, 30 individual aphids were collected and transferred into a microfuge tube. Aphids were washed with 1 ml of 75% ethanol by gently shaking for 60 s. The insects were then stored in −80 °C for DNA extraction.

Extraction of genome DNA

Total genomic DNA from a sample was extracted using a QIAampDNA Micro Kit (OMEGA Forensic DNA Kit) following the directionsprovided by the

115   manufacturer. DNA concentration and purity was monitored on a 1% agarose gel.

116   DNA concentration was diluted to 1ng/μL with sterile water and used as template for

117   PCR amplification.

Amplificon generation

119   The variable regions 4-5 (16SV4-V5) in the internal transcribed spacers (ITS) of

120   16S rRNA genes were amplified using a pair of specific primers, namely 515F "5-

121   GTGCCAGCMGCCGCGG-3" and 907R"5- CCGTCAATTCMTTTRAGTTT-

122   3",with barcodes. All PCR reactions were carried out with Phusion® High-Fidelity

123   PCR Master Mix (New England Biolabs). A PCR reaction mix contained 5 μl 5x

124   reaction buffer, 5 μl 5x GC buffer, 2 μl dNTP (2.5 mM), 1 μl Forward primer and Reverse

125   primer, respectively (10 mM), 2 μl DNA Template, 8.75 μl ddH2O, and 0.25 μl Q5 DNA

126   Polymerase. PCR amplification was achieved by an initial denaturation at 98℃for 2min, followed

127   by 25-30 cycles of denaturation (98℃ for 15s) , annealing (55℃ for 30s) , and extension (72℃

128   for 30s). A final extension step at 72℃ for 5 min was included at the end of each PCR

129   amplification.

PCR amplification and sequencing

131   PCR products were mixed with the same volume of 1x loading buffer (containing

132   SYBR green) and analyzed on a 2% agarose gel. Samples with a main 400-450bp

133   band were purified with a Qiagen Gel Extraction Kit (Qiagen, Germany). Amplicons

134   were then quantified and sequencing libraries were generated using NEB Next Ultra

135   DNA library Prep Kit for Illumina (New England Biolabs Inc. USA) according to

136   manufacturer's recommendations. The amplicon libraries were subsequently

137   sequenced on an Illumina MiSeq platform at Personalbio (Shanghai, P.R. China).

138   Bioinformatics analyses

139   Paired-end reads were merged using FLASH [19]. Low quality reads were

140   removed. Sequences were analyzed with the QIIME software package [20] using

141   default parameters for each step. The Uparse software [21] was used to cluster the

142   sequences into operational taxonomic units (OTUs) at an identity threshold of 97%.

143   The RDP Classifier [22] was used to assign each OTU to a taxonomic level. Other

144   analyses, including rarefaction curves, Shannon index, and Good's coverage, were

145   performed with QIIME. In addition, the OTU table produced by the QIIME pipeline

146   was imported into MEGAN 4 and mapped on the GreenGene Database [23, 24].

147   Principal Coordinate Analysis (PCoA) was performed to get principal

148   coordinates and visualize from complex, multidimensional data. PCoA has been

149   recognized as a simple and straight-forward method to group and separate samples in

150   a dataset. In this study, PCoA was used to analyze the sequencing results using

151   WGCNA package, stat packages and ggplot2 package in R software.

152   Statistical methods

153   All experimental results were evaluated using analysis of variance (ANOVA) for

154   multiple comparisons followed by the Turkey test. Differences were considered

155   significant at p < 0.05. All analyses were done using the programs WGCNA, STAT,

156   and ggplot2 in the R software package.

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Results

Sequence reads and OTUs

In total, 975,583 raw reads were obtained from all samples, and after filtering, 973,373 high-quality sequences were retained and subsequently clustered into 194 OTUs (S1 Table). Of the 194 OTUs, five were classified as Archaea, 83 were Bacteria, and the remaining 106 remain to be determind since they showed no blast hits. Of the 82 bacterial OTUs, 56 (67.5%) were Proteobacteria, 10 belong to Firmicutes, and the remaining 17 belong to Cyanobacteria and others.

The distribution of reads and OTUs duringdifferent seasons is shown in Table 1, along with other parameters including Chao1 Index, Shannon Index, and Good's coverage. The highest number of reads was obtained in the sample collected on October 19, but the highest number of OTUs was obtained in the sample collected on March 24. In comparison, the lowest number of reads was obtained in the sample collected on May 27, whereas the lowest number of OTUs was obtained in the sample collected on September 16.

Major and minor OTUs

The 194 OTUs were divided into six groups based on their highest number of sequence reads at any time point (Table 2). Group 1 contains only one OTU, which is highly abundant with sequence reads over 30,000 in all samples at different time points. This OTU belongs to Buchnera, strongly suggesting that this is the principal endosymbiont of *Schlechtendalia chinensis*. Group 2, which comprises also highly abundant taxa, contains six OTUs with the highest numbers of reads between 100 to

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180 2,000 per sample. The six OTUs belong to different taxonomic units including

181 *Bacillus*, *Limnohabitans*, *Candidatus Cloacamonas*, *Comamonadaceae* and

182 *Pseudomonas*. Group 3, defined here as medium-abundant OTUs, contains 38 OTUs

183 with the highest numbers of reads between 10 to 100 in different samples. Twenty-

184 five OTUs had no blast hit, whereas the remaining OTUs corresponded to different

185 taxa and are listed in the table 2. Group 4, which represent low- abundant taxa,

186 contains 47 OTUs with the highest read numbers between 5 and 10 in different

187 samples. Twenty-eight OTUs had no blast hit, whereas the remaining OTUs

188 corresponded to different taxonomic units. Group 5, comprisesinfrequent OTUs,

189 contains 79 OTUs with the highest numbers of reads only between 2 to 5 in different

190 samples, whereas group 6, referred as rare OTUs, contains 22 OTUs with the highest

191 number of reads below 2.

192 ## OTUs associated that differ between host plants

193 Samples collected in March, April, and November were isolated from the host

194 moss, whereas the remaining samples were obtained from Sumac galls. There were 12

195 OTUs found exclusively in aphids collected from moss, and 11 OTUs were present

196 exclusively in aphids collected from Sumac galls. The dynamic changes at different

197 sampling time points of these OTUs specific to aphids feeding on the two different

198 hosts are shown in Figs 1 and 2. The OTUs specific to aphids on different host plants

199 were also listed in Table 3. There are five genera that were detected only in moss-

200 residing aphids. These, included *Wolbachia*, a genus from the family Gemmataceae, a

201 genus from the family Pirellulaceae, *Phormidium*, and *Streptococcust*. And there are

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202 four genera (family) that were detected only in aphids feeding in Sumac galls,

203 including *pGrfC26*, a genus from the family Xenococcaceae, a genus from the

204 phylum Crenarchaeota, and *Nitrosopumilus*.

205   The OTUs were roughly equally present in aphids feeding on the two different

206 hosts (Table 3). There are nine OTUs from different genera or families that were

207 always present in aphids collected at different time points, including *Buchnera*,

208 *Stenotrophomonas*, *Acinetobacter*, *Bacillus*, *Pseudomonas*, *Streptophyta*,

209 *Anoxybacillus kestanbolensis*, Rhodococcus, and Comamonadaceae. The remaining

210 OTUs were detected in aphids residing on either host plant, but their presence was not

211 consistent at all time points.

Beta diversity of microbiota during the whole life cycle of *S. chinensis*

214 A weighted UniFrac principal coordinates analysis (PCoA) was performed to

215 compare the overall structure of microbiota from all samples based on the relative

216 abundance of OTUs (Fig 3). There was an obvious separation between samples from

217 different months even though some unevenly distributed presence. PC1, PC2 and PC3

218 accounted for 42.81%, 22.28% and 15.47% of total variations, respectively. This

219 analysis showed differences in beta diversity resulted in separation between aphids

220 feeding on moss and Sumac galls.

Discussion

222   The most abundant OTU was denovo496, which accounted for 80-99% of total

223 sequence reads in all samples (S1 Table, S1 Fig). The taxonomy of denovo496 is

224   *Buchnera*, which is an endosymbiont in all aphid species [3, 25-30]. (3, 25, 26, 27, 28,

225   29, 30) Since *Buchnera* is an obligate endosymbiont and is present in every aphid

226   cell, it is not surprising that denovo496 was the most abundant microbe detected in

227   our study. The abundance of sequence reads for *Buchnera* suggests that our sequence

228   data were reliable and representative. Interestingly, there were 17 other OTUs

229   corresponding to *Buchnera* in addition to denovo496 (S1 Table). The 17 OTUs were

230   much less abundant with average sequence reads equal or below 16. However, many

231   of these minor *Buchnera* OTUs, for example the OTU denovo915 (S1 Fig.), were

232   consistently distributed in different samples, suggesting that these minor OTUs were

233   not caused by sequence errors or other artifacts. The exact sources for these minor

234   *Buchnera* OTUs remain to be determined. The minor OTUs could be due to minor

235   alleles among *Buchnera* individuals in aphid cells.

236   The second-most abundant OTU was denovo743, which we detected in all

237   samples across different time points. The percentages of denovo743 reads were below

238   0.8% of total reads in aphids feeding on moss, but the percentages reached as high as

239   7.5% of total reads in aphids living in galls. The taxonomy of denovo743 is

240   *Streptophyta* from chloroplasts. Based on its taxonomy and abundance distribution,

241   the sequences corresponding denovo743 were likely due to contamination of

242   chloroplast DNA. It could be due to chloroplasts from gall tissues that stuck to the

243   outside of aphid bodies. Alternatively, chloroplasts from gall tissues were sucked up

244   to aphid guts.

245   Aside from *Buchnera*, other major bacteria associated with *S. chinensis* were

246   from the genera *Bacillus*, *Limnohabitans*, *Candidatus Cloacamonas*, a genus from the

247   family Comamonadacease, and *Pseudomonas* (Table 2, S1 Table). *Bacillus* is found

248   commonly in aphids [31] and in other insect species [2, 32, 33, 34]. *Bacillus* protects

249   the host from other bacterial and fungal colonization by producing antimicrobial

250   compounds such as phenols [33, 35]. *Pseudomonas* bacteria colonize a wide range of

251   ecological niches [27, 36], but it is not clear what kind of benefits this bacterium

252   provides to aphids.

253   We detected 23 OTUs detected in all aphid samples collected from either moss or

254   Sumac galls. Even though they were common to all samples, the abundance of these

255   OTUs varied greatly from sample to sample exceptfor *Buchnera*. Therefore, it is not

256   clear if these common OTUs are obligate or facultative and further research is needed

257   to reveal their impact on the aphid host or aphid - plant interactions.

258   There were 12 OTUs unique to moss-residing aphid populations (Table 3).

259   Typically, each OTU was detected at only one time point, either in November or

260   March except the OTU denovo1288, which was found in Nov, Mar, and Apr samples

261   (Fig 1). The presence of a specific symbiont at a specific time point indicated that

262   these symbionts are likely facultative and play transient roles in the aphid life cycle.

263   *Wolbachia* was among these moss-specific OTUs. *Wolbachia* has been previously

264   found in aphids and it is a facultative symbionts that plays a role in the reproduction

265   of its host insects [37, 38], as well as a role in host plant fitness [38]. Other symbionts

266   specific to moss-residing aphids included bacteria from the families or genera

267   Comamonadaceae, *Phormidium,* Pirellulaceae, Gemmataceae, and *Streptococcust*.

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268   There were 11 OTUs specific to aphids associated with Sumac galls (Table 3).

269   Most of these were present (or mainly present) in samples from a single time point

270   (Fig 2), suggesting that they are likely facultative in their interaction with the host

271   aphid. *Crenarchaeota* was among the symbionts unique in aphids collected from

272   Sumac galls. *Crenarchaeota* symbionts have been reported to be involved nitrogen

273   cycling [39]. *Nitrosopumilus* was another symbiont specific to aphids feeding in

274   Sumac galls. *Nitrosopumilus* has been found to play roles in ammoxidation and CO_2

275   assimilation in insects [40, 41].

276   In summary, a comprehensive survey was conducted on microbes associated with

277   *S. chinensis* aphids on two different hosts at different time points. This survey

278   identified a range of OTUs from highly abundant to rare microbes from different

279   samples. We discovered both common and plant-specific symbionts. Identification of

280   these symbionts provides a foundation for future studies to determine the roles of

281   different symbionts on aphid physiology and insect - plant interactions.

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Table 1. Distribution of OTUs among samples collected on different time.

Sample date	11/30	3/24	4/14	5/27	6/10	7/09	8/12	9/16	10/19
Clean reads	98,653	104,744	111,689	86,722	117,146	117,901	100,097	117,142	119,279
OTU	98	146	131	128	82	145	119	64	87
Chao1 Index	113.1667	164.0714	156.0714	143	94.83333	153	139.7143	79	96.1
Shannon Index	0.549917	0.267498	0.198749	0.449417	0.246772	0.711832	0.154277	0.103239	0.108371
Good's coverage	0.99986	0.99977	0.99973	0.9997	0.99978	0.99984	0.9997	0.99985	0.99986

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Table 2. Groups of associated microbes with different levels of sequence reads. The lowest known level of taxonomy is given in the table. The numbers
in parenthesis are the numbers of OTUs in that taxonomic unit.

Group	Sequence reads	OTUs	OTU taxonomy
1. Super abundant OTU	>30,000	1	Buchnera (1)
2. Highly abundant OTUs	<2,000 - 100	6	Bacillus (1), Limnohabitans (1), Candidatus Cloacamonas (1), Comamonadaceae (1) Pseudomonas (2)
3. Medium Abundant OTUs	<100 - 10	38	Comamonas (1), Lactobacillales (1), Buchnera (2), Anaerolinaceae (1), Stenotrophomonas (1), Pirellulaceae (1), Rhodococcus (1), Streptococcus (1), Acinetobacter (1), Thermus (1), Crenarchaeota (1), Comamonadaceae (1), no blast hit (25)
4. Low abundant OTUs	<10 - 5	47	Xanthomonadaceae (1), Bacillus (2), Bacteria (1), Gemmataceae (1), Rhodobacter (1), Aminobacterium (1), Nitrosopumilus (1), Sphingomonas(1), Archaea (2), Pseudoxanthomonas (1), Buchnera (1),Anaerolinaceae (1), Methyloversatilis (1), Propionibacterium (1), Leuconostoc (1), Oxalobacteraceae (1), Anaerolinaceae (1), no blast hit (28)
5. Infrequent OTUs	<5 - 2	79	Buchnera (7), Alteromonadales (1), Comamonadaceae (7), Phormidium (1), Allochromatium (1), Rhizobium (1), Staphylococcus (1), Anoxybacillus kestanbolensis (1), Mycoplana (1), Archaea (1), Xenococcaceae (1), Enterobacteriaceae (3), Streptophyta (1), Bacillales (1), Pseudomonas stutzeri (1), Streptophyta (2), Acinetobacter (1), Halomonas (1), Methylobacteriaceae (1), Brevibacillus reuszeri (1), Roseomonas rosea(1), Caulobacteraceae (1), Burkholderiales (1), Rhizobiales (1), Wolbachia (1), no blast hit (39)
6. Rare OUTs	<2 - 1	22	Buchnera (5), Thauera (1), Brevundimonas diminuta (1), Enhydrobacter (1), no blast hit (14)

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Table 3. OTUs associated with aphids feeding on two different host plants.

| Host Plant	OTUs	Taxonomy (OTU name)					
Exclusively in aphids from moss	12	No blast hit (denovo120)	*Wolbachia* (denovo1288)	Gemmataceae (denovo965)	No blast hit (denovo553)	Pirellulaceae (denovo281)	Comamonadaceae (denovo1605)
		Phormidium (denovo1382)	No blast hit (denovo1794)	No blast hit (denovo1959)	No blast hit (denovo1881)	No blast hit (denovo326)	*Streptococcust* (denovo1937)
Exclusively in aphids from Sumac	11	*pGrfC26* (denovo1719)	Xenococcaceae (denovo1801)	*Buchnera* (denovo160)	No blast hit (denovo1817)	No blast hit (denovo1672)	No blast hit (denovo316)
		No blast hit (denovo536)	*Crenarchaeota* (denovo867)	*Nitrosopumilus* (denovo1892)	*Streptophyta* (denovo194)	No blast hit (denovo1755)	
Present in aphids from all samples	23	*Buchnera* (denovo528)	*Stenotrophomonas* (denovo2048)	*Buchnera* (denovo915)	*Acinetobacter* (denovo1435)	*Bacillus* (denovo187)	No blast hit (denovo780)
		No blast hit (denovo2067)	*Pseudomonas stutzeri*(denovo739)	*Buchnera* (denovo2027)	*Pseudomonas* (denovo1118)	*Buchnera* (denovo1712)	*Buchnera* (denovo1556)
		Buchnera (denovo753)	*Buchnera* (denovo496)	No blast hit (denovo1629)	*Streptophyta* (denovo743)	Buchnera (denovo155)	*Anoxybacillus kestanbolensis* (denovo1177)
		Rhodococcus (denovo1058)	Buchnera (denovo104)	Comamonadaceae (denovo544)	Buchnera (denovo1489)	No blast hit (denovo1464)	
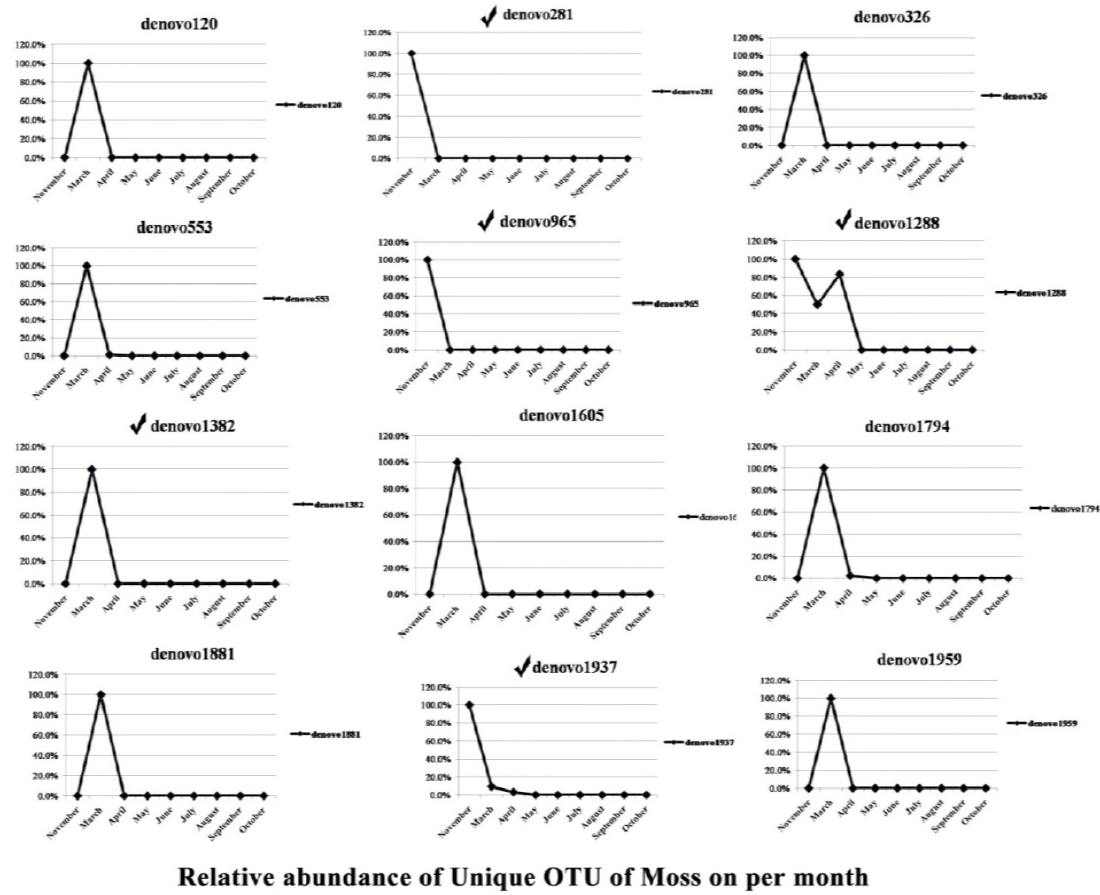
Figure 1. Dynamic changes at different sampling times of OTUs specific to aphids feeding on moss (from Nov to Apr). The symbol ✓ represents these OTUs that were classified into genus or family. The remaining OTUs had no blast hits.
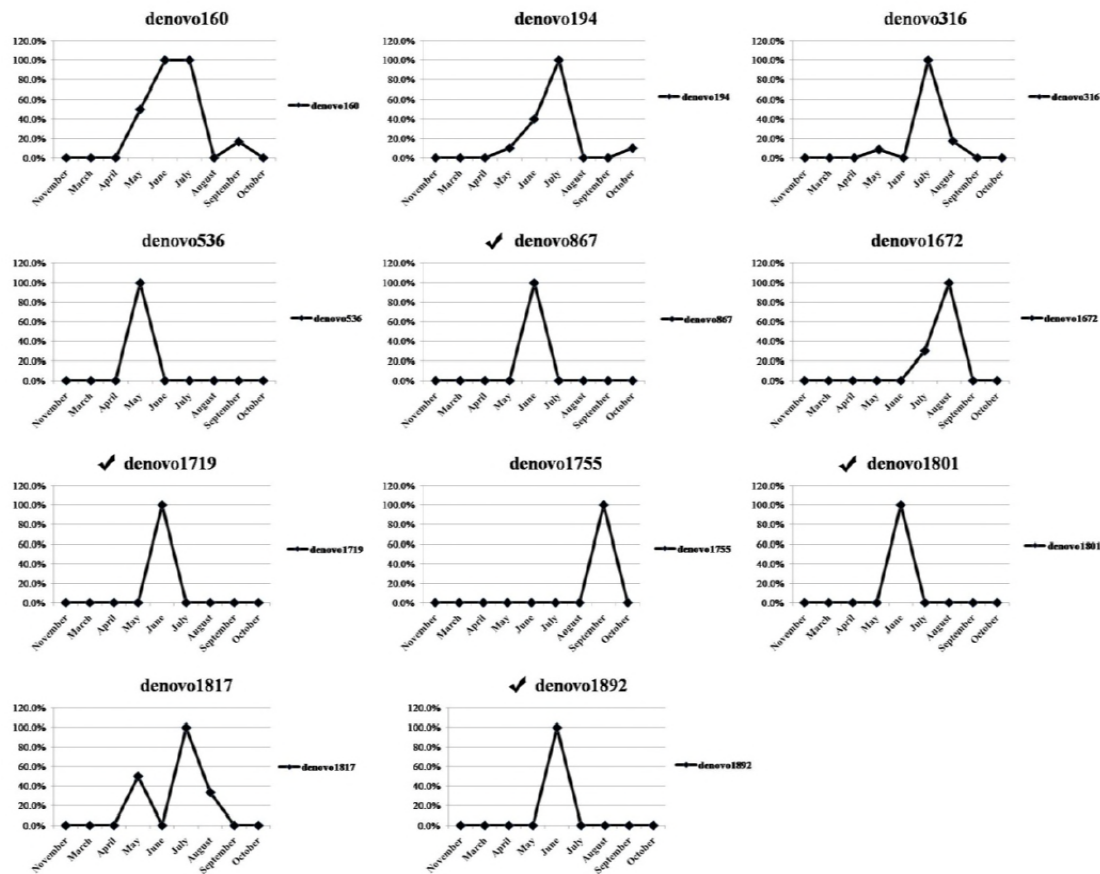
Relative abundance of Unique OTU of Gall on per month

Figure 2. Dynamic changes at different sampling times of OTUs specific to aphids feeding in galls on Sumac trees (May to Oct when aphids lived in galls). The symbol ✓ indicates the OTUs that were classified into genus or family. The remaining OTUs had no blast hits.
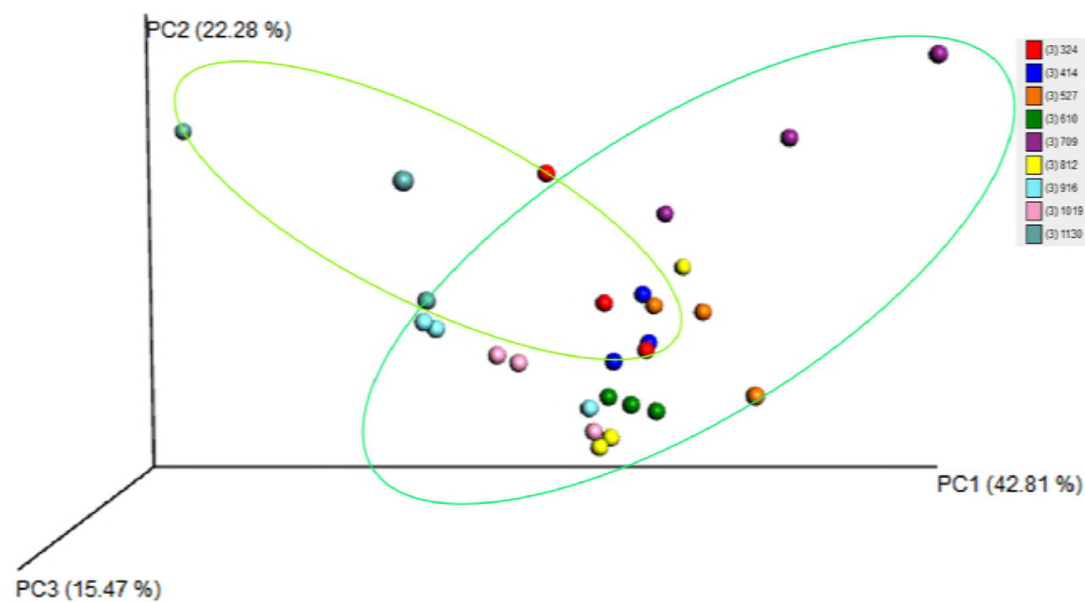
Figure 3. Weighted UniFrac principal coordinates analysis (PCoA) plots based on weighted UniFrac metric. Light green circle indicates data from aphids feeding on the moss. Dark green circle indicates data from aphids feeding in galls on Sumac trees.
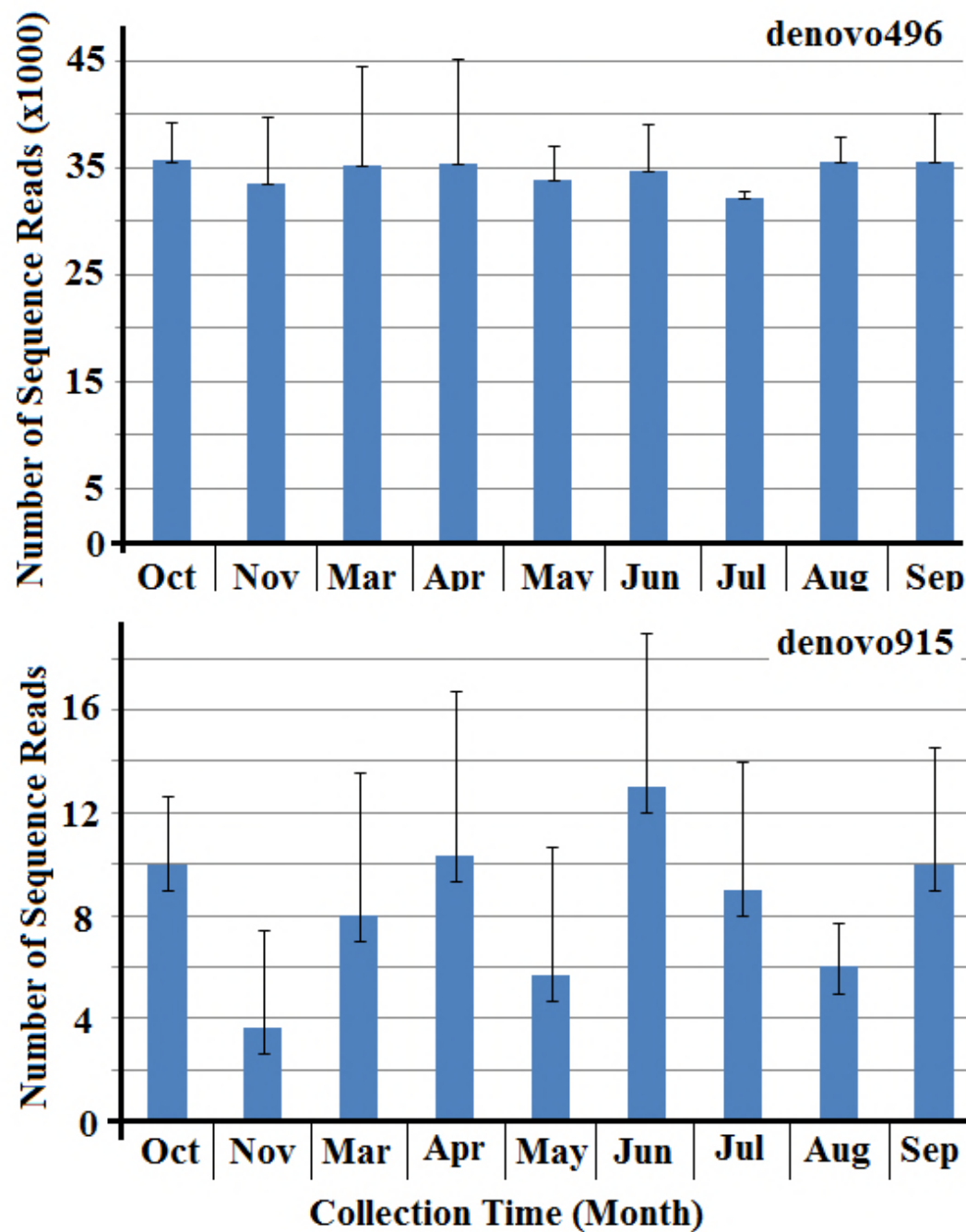
Figure S1. Abundance and variation of sequence reads for the two OTUs, denovo496 and denovo915, in different samples collected at different time points. The taxonomy of both OTUs correspond to *Buchnera*, an obligate endosymbiont. Standard errors are shown on the top of each bar.