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Discovery of a new *Wolbachia* supergroup in cave spider species and the lateral transfer of phage WO among distant hosts

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ABSTRACT

Wolbachia are widespread intracellular bacteria infecting the major classes of arthropods and some filarial nematodes. In arthropods, *Wolbachia* have evolved various intriguing reproductive manipulations, including cytoplasmic incompatibility, parthenogenesis, feminization, and male killing. Sixteen supergroups of *Wolbachia* have been identified, named A–Q (except G). Though *Wolbachia* present great diversity in arthropods, spiders, especially cave spiders, are still a poorly surveyed group of *Wolbachia* hosts. Here, we report a novel *Wolbachia* supergroup from nine *Telema* cave spiders (Araneae: Telemidae) based on five molecular markers (*16S rRNA*, *ftsZ*, *gltA*, *groEL*, and *coxA*). In addition, phage WO, which was previously reported only in *Wolbachia* supergroups A, B, and F, infects this new *Wolbachia* supergroup. We detected a 100% infection rate for phage WO and *Wolbachia* in *Telema* species. The phylogenetic trees of phage WO and *Wolbachia* are not congruent, which suggests that horizontal transfer of phage WO has occurred in these secluded species. Additionally, these data indicate *Telema–Wolbachia*-phage WO may be a good model for exploring the horizontal transfer history of WO among different host species.

1. Introduction

Wolbachia are maternally inherited rickettsial endosymbiotic bacteria in the class Alphaproteobacteria and are one of the most widespread obligate intracellular bacteria in some classes of arthropods and nematodes (Ferri et al., 2011; Hilgenboecker et al., 2008; Jeyaprakash and Hoy, 2000; Werren, 1997). A meta-analysis has suggested that 40% of insect species are infected with *Wolbachia* (Zug and Hammerstein, 2012). Wolbachia use an arsenal of reproductive manipulations in hosts, including feminization (Legrand et al., 1987), parthenogenesis (Stouthamer et al., 1990), male killing (Werren et al., 1994), and cytoplasmic incompatibility (Breeuwer and Werren, 1990). These phenotypes contribute to increasing the frequency of infected females in a host population, thus propagating *Wolbachia* worldwide.

Wolbachia are highly divergent and have been divided into 16 supergroups (A–Q, except for G, which is a combination of A and B) (Augustinos et al., 2011; Baldo et al., 2007; Bing et al., 2014; Bordenstein and Rosengaus, 2005; Glowska et al., 2015; Haegeman et al., 2009; Lo et al., 2002; Ros et al., 2009). The *Wolbachia* supergroups are classified mainly based on the genetic distance of the molecular markers *16S rRNA*, *gltA* (encoding citrate synthase), *groEL* (encoding heat-shock protein 60), *coxA* (encoding cytochrome c oxidase), *ftsZ* (encoding cell division protein), and *wsp* (encoding *Wolbachia* surface protein) (Casiraghi et al., 2005; O'Neill et al., 1992; Werren and Windsor, 2000). *Wolbachia* genotyping is inferred from multi locus sequence typing (MLST) of genes (*gatB*, *coxA*, *hcpA*, *fbpA*, and *ftsZ*) and the four hypervariable regions of WSP protein (Baldo et al., 2006, 2005).

Genome reduction is the predominant evolutionary trend in obligate intracellular bacteria and most are bacteriophage absent, like *Buchnera* (Moran and Bennett, 2014; Shigenobu et al., 2000). In *Wolbachia*, phage WO is widespread, with about 89% *Wolbachia* strains harboring WO (Bordenstein and Wernegreen, 2004). However, almost all of the phage WO infections are within *Wolbachia* supergroups A, B, and F. Based on genomic analyses, *Wolbachia* supergroups C and D have lost phage WO (Darby et al., 2012; Foster et al., 2005). Whether phage WO plays some role in *Wolbachia* reproductive manipulation or can be developed to be a genetic vector for *Wolbachia* research are two hot topics (Kent and Bordenstein, 2010). Indeed, some sex specific (Sinkins et al., 2005) and stage-specific expression of WO genes (Sanogo and Dobson, 2006; Wang et al., 2014) has been shown, which indicates that these genes may play an active role in *Wolbachia* biology. Phage WO is a temperate phage that can transfer within and between discrete *Wolbachia* supergroups (A and B) (Gavotte et al., 2004; Kent et al., 2011), which suggests that phage WO might mediate gene transfer. However, we still have little knowledge of whether other *Wolbachia* supergroups are infected with phage WO, and if yes, whether phage WO

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can transfer between more discrete *Wolbachia* supergroups except between previously reported supergroups A and B.

Although *Wolbachia* is well characterized in insects, there are few reports of *Wolbachia* diversity in spiders. Studies have shown that some spiders are infected with *Wolbachia* belonging to supergroups A and B (Duron et al., 2008a; Goodacre et al., 2006; Lo et al., 2007; Rowley et al., 2004; Vanthournout et al., 2011). In the present study, we address *Wolbachia* diversity in cave spiders. Cave environments are regarded as an extreme habitat characterized by cool and moist air, permanent darkness, scarce energy sources, and constant environment compared with surface conditions (Gabriel and Northup, 2013; Zhang and Li, 2014). Usually, endosymbionts are critical for host adaptation and survival in cave environments, and there are novel endosymbionts compared to the external biota (Morse et al., 2012; Paoletti et al., 2013). Thus, *Wolbachia* diversity in cave spiders may have been affected by long-term isolation from external *Wolbachia* strains. In this study, we examine the distribution of *Wolbachia* in *Telema cordata* and eight species of the *Telema cucurbitina* "species complex" collected from Guangxi Province, China. All of these spider species are typical cave spiders. We not only detected 100% prevalence of *Wolbachia* and phage WO in these *Telema* species, but also identified that the *Wolbachia* infecting the *Telema* species belong to a novel supergroup (supergroup R). In addition, we reveal the horizontal transfer of phage WO among the distant *Wolbachia* supergroups A and R.

2. Materials and methods

2.1. Spider collection and identification

All of the spiders used in the study (see Table 1) were collected in Guangxi Province, China from April to July, 2013. The specimens were carefully morphologically identified, and the given names were cited from Zhang et al.'s study (Zhang and Li, 2014). All identified spiders were initially immersed in 95% ethanol and subsequently maintained at −20 °C until DNA extraction.

2.2. Isolation, amplification, and sequencing of genomic DNA

Before DNA extraction, each specimen was washed several times with 70% ethanol followed by sterile water to remove surface contaminants. DNA was isolated from each spider using an EasyPure Genomic DNA extraction kit (TransGen, Beijing, China) following the manufacturer's recommendations. The quality of the DNA templates was confirmed by the amplification of a partial fragment of cytochrome *c* oxidase subunit I with the primers LCO1490 and HCO2198 (see Table S1) (Vrijenhoek, 1994). DNA templates of poor quality were discarded.

All species (Table 1) were screened for the presence of *Wolbachia* strains by amplification of 16S *rRNA* and/or *wsp* using the primers shown in Table S1. When *Wolbachia* were identified, additional PCR was carried out based on *Wolbachia* protein-coding genes (*ftsZ*, *gltA*, *groEL*, *coxA*, *gatB*, *fbpA*, and *hcpA*) and phage WO minor capsid gene *orf7* (Table 1). All the primers are shown in Table S1.

The PCR program was 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 40 s at 47–60 °C, and 25 s at 72 °C; and 10 min at 72 °C for the final extension step. Negative controls with sterile water as template were used for all PCR experiments. The PCR components were added as recommended by the manufacturer of TransTaq DNA Polymerase HiFi Fidelity (TransGen, Beijing, China). The PCR products were electrophoresed using 1% agarose gels in Tris-CH_3COOH buffer. Following electrophoresis, the gels were dyed with GelStain (TransGen, Beijing, China) and imaged on a VILBER FUSION FX5 (Vilber Lourmat, France). If there was a single amplified band, the PCR products were purified with the EasyPure PCR purification kit (TransGen, Beijing, China) and directly sequenced with an ABI 3730 sequencer (Biosune, Beijing, China). If there were more than one amplification band, the expected band was excised from the gels and purified with the EasyPure Quick Gel PCR purification kit (TransGen, Beijing, China) and cloned with the Peasy-T5 vector (TransGen, Beijing, China); a minimum of three positive clones were sequenced. For each gene, at least two specimens per species were sequenced. All de novo nucleotide sequences in this study were deposited in GenBank under accession numbers KT319068–KT319104 and KU057803–KU057809.

2.3. Sequence analyses

We used representative *Wolbachia* supergroups described in the literature to classify *Wolbachia* strains from *Telema* species. The representative strains for each *Wolbachia* supergroup were chosen for which at least two of the five *Wolbachia* loci (*16S rRNA*, *ftsZ*, *gltA*, *groEL*, and *coxA*) were available from the GenBank (http://www.ncbi.nlm.nih.gov/genbank/). We translated the protein coding sequences into amino acid sequences by the TranslatorX version 1.1 (Abascal et al., 2010). Then we aligned the protein sequences using MUSCLE

Table 1
Screening of *Wolbachia* and phage WO in *Telema* species.

Telema species	Location[a]	*16S rRNA*	*fbpA*	*gatB*	*hcpA*	*gltA*	*groEL*	*coxA*	*ftsZ*	*wsp*	*orf7*	No. of specimens	*Wolbachia* infection rate (%)	WO infection rate (%)
Telema cucurbitina complex sp. Cave_num1	Lingui country (25°12.819′N, 110°12.050′E)	+	+	−	−	−	−	+	−	+	+	12	100	100
Telema cucurbitina complex sp. Cave_num2	Liuzhou city (24°13.782′N, 109°24.663′E)	+	+	−	−	−	−	+	−	+	+	11	100	100
Telema cucurbitina complex sp. Cave_num6	Yangshuo country (24°56.686′N, 110°36.369′E)	+	+	−	+	−	−	+	+	−	+	10	100	100
Telema cucurbitina complex sp. Cave_num7	Laibin city (22°43.648′N, 109°05.447′E)	+	+	−	−	−	−	+	−	−	+	15	100	100
Telema cucurbitina complex sp. Cave_num10	Xiangzhou country (23°57.278′N, 109°39.696′E)	−	−	−	−	−	−	−	−	+	+	3	100	100
Telema cucurbitina complex sp. Cave_num11	Lingchuan country (25° 18.575′N, 110° 13.875′E)	+	−	−	−	−	−	+	−	+	+	13	100	100
Telema cucurbitina complex sp. Cave_num14	Guizhou city (25° 16.33′N, 110° 18.25′E)	+	−	−	−	−	−	+	−	+	+	4	100	100
Telema cucurbitina complex sp. Cave_num15	Liuzhou city (24° 13.782′N, 109° 24.663′E)	+	−	−	−	−	−	+	−	+	+	3	100	100
Telema cordata	Xiangzhou country (23° 57.278′N, 10° 39.696′E)	+	+	−	+	−	+	+	+	+	+	16	100	100

+: positive amplification,
−: failure to detect amplification product.
[a] All of the places are in Guangxi Province, China.

and subsequent back translation into codon sequences. The *16S rRNA* sequences were aligned using Infernal, a stochastic context-free grammar-based aligner (http://rdp.cme.msu.edu/) (Cole et al., 2014). We created a supermatrix of the five alignments (*16S rRNA*, *coxA*, *ftsZ*, *gltA*, and *groEL*) with FASconCAT version 1.0 (Kuck and Meusemann, 2010), and the nucleotide acid was replaced with "N" if we failed to obtain the gene sequence in the *Wolbachia* strain. We determined the best partitioning models for each of the genes with Partition Finder version 1.1.1 (Lanfear et al., 2012). The best partitioning model for *Wolbachia* concatenated genes (*16S rRNA*, *coxA*, *ftsZ*, *gltA*, and *groEL*) was in Table S2. We used *Anaplasma marginale* and *Ehrlichia ruminantium* as outgroups.

For WO *orf7* gene, jModelTest 2 (Darriba et al., 2012) was used to determine the best evolutionary model based on the corrected Akaike information criterion (AICc). PhyML 3.0 (Guindon et al., 2010) or RAxML v8 (Stamatakis, 2014) was used to build Maximum Likelihood (ML) phylogenetic trees, and MrBayes 3.2 (Ronquist et al., 2012) was used to build phylogenetic trees with Bayesian Inference (BI) methods.

To evaluate the topological congruence, we employed the Shimodaira–Hasegawa test (SH-test) (Shimodaira and Hasegawa, 1999) in RAxML v8 ("-f h" option) (Stamatakis, 2014) for statistical support. One paired constraint tree generated from the ML analysis (phage WO and their *Wolbachia* hosts, 1000-bootstrap trees generated) was compared by the SH-test.

Potential recombination events for *16S rRNA*, *coxA*, *ftsZ*, and *groEL* were done for individual and concatenated alignments, including *gltA*, using the recombination detecting program RDP4 v.4.24 by all algorithms (RDP, Genconv, Maxchi, Chimera, Siscan LARD, BootScan and 3Seq) (Martin et al., 2010) and Phi test in SplitsTree4 (Bruen et al., 2006). We used the default options for all analyses.

3. Results

3.1. Prevalence of Wolbachia and bacteriophage WO in Telema species

Screening of 87 individuals of the nine *Telema* species showed that the infection rates of *Wolbachia* and phage WO are 100% for all species.

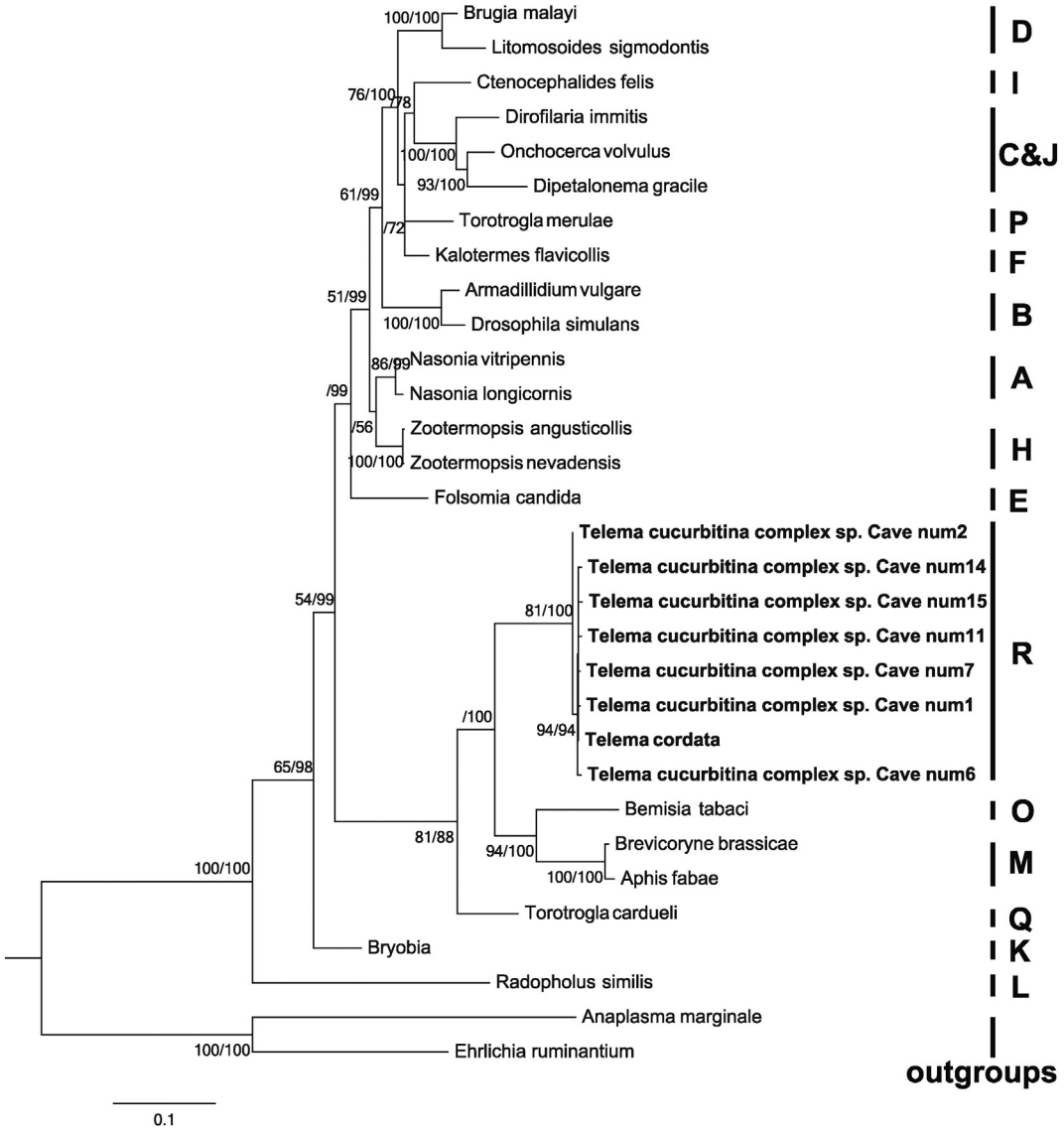

Fig. 1. Phylogenetic tree based on a supermatrix of five *Wolbachia* loci (*16S rRNA*, *coxA*, *ftsZ*, *gltA*, and *groEL*). All previously reported *Wolbachia* supergroups for which at least two of the five *Wolbachia* loci (*16S rRNA*, *coxA*, *ftsZ*, *gltA*, and *groEL*) were available were used in this analysis. Maximum Likelihood (ML) and Bayesian inference methods were utilized. ML bootstrap values based on 1000 replicates and Bayesian posterior probabilities are depicted as numbers beside each branch (the first number is ML value and the later one is Bayesian probability). Only values larger than 50% are shown. The *Wolbachia* strains are characterized by the names of their host species. New *Wolbachia* from spiders described in this study are highlighted in bold. Capital letters indicate previously reported *Wolbachia* supergroups and the novel supergroup R.

Screening was based on both *16S rRNA* and/or *wsp* PCR assays for *Wolbachia* and *orf7* PCR assays for phage WO (Table 1).

3.2. Phylogenetic analysis of spider Wolbachia strains indicating a novel supergroup

We used five *Wolbachia* markers, including *16S rRNA*, *gltA*, *groEL*, *coxA*, and *ftsZ*, for the *Wolbachia* phylogenetic analysis (Table S3). We excluded *gatB*, *fbpA*, and *hcpA* for further analysis, as we obtained few PCR products from selected host species (see Table 1). These results indicate that there might be too many mismatches on priming regions and that the *Wolbachia* in these spiders are genetically divergent. Analyses of these five loci (3241 bp in total) reveals that *Telema* species (For *Telema cucurbitina* complex sp. Cave_num10, we obtained few sequences. Thus, we did not analyze this species here. See Discussion section) are infected by one *Wolbachia* supergroup that is divergent from other previously defined supergroups, which we propose to name supergroup R (Fig. 1). Phylogenetic tree analyses show that *Wolbachia* strains in each of the supergroups, represented by more than one strain, are monophyletic, although the relationships between the supergroups are still unclear (Fig. 1). In the single gene analyses, all *Wolbachia* genes from *Telema* are again recovered as monophyletic, except one gene from *T. cucurbitina* complex sp. Cave_num15, which clusters with *Wolbachia* supergroup B (*16S rRNA*) and one gene from *T. cucurbitina* complex sp. Cave_num2, which clusters with *Wolbachia* supergroup A (*coxA*) (Fig. S1). These results indicate we may not infer phylogenetic relationship just based on one gene and support the existence of the new *Wolbachia* supergroup R. We tested recombination events for alignments of individual *16S rRNA*, *coxA*, *ftsZ*, *groEL* and concatenated with *gltA* using RDP4 v.4.24 (Martin et al., 2010) and Phi test in SplitsTree4 (Bruen et al., 2006). No indication of significant recombination events was detected.

3.3. Lateral transfer of bacteriophages WO among different Wolbachia supergroups

Based on the analyses of WO minor capsid gene *orf7*, we demonstrate lateral transfer of WO between different *Wolbachia* supergroups of A and R. First, we detected identical *orf7* sequences in distantly related *Wolbachia* supergroups (*orf7* in phages of WOVitA2 and WOCor1from supergroups A and R) (Fig. 2). Second, the phage WO phylogenetic tree based on *orf7* (Fig. 2) does not correlate with the *Wolbachia* phylogeny (Fig. 1). We compared the ML phylogenies of phage WO and *Wolbachia* using the SH-test (Shimodaira and Hasegawa, 1999) to statistically examine the topological incongruence (Fig. 3). For the phylogenetic tree of *orf7*, we chose only one *orf7* sequence if more than one phage WO haplotypes infects the same *Wolbachia* strain and precluded the duplicate *orf7* sequence in *Wolbachia* of *Telema* species, as most of these *Wolbachia* strains harbor identical *orf7* sequence (Fig. 2). The phage WO phylogenetic tree has significant differences compared to the *Wolbachia* phylogenetic tree ($p < 0.01$, D (LH) = 92.90, SD = 14.90, SH-test), which indicates a strong topological incongruence between bacteriophage WO and *Wolbachia* (Fig. 3).

4. Discussion

4.1. Increasing our knowledge of Wolbachia in spiders

Considering the high incidence of *Wolbachia* in insects, the infection of *Wolbachia* in the spiders is also worthy of investigation. However, there have only been a few reports of *Wolbachia* in spiders, which found that these spiders contained *Wolbachia* strains of supergroups A and B that commonly infect insects (Baldo et al., 2008; Duron et al., 2008b; Rowley et al., 2004; Woo Oh et al., 2000). There have been no previous reports of the *Wolbachia* in the cave spiders, which live in a long-term isolated environment. The *Wolbachia* diversity in these spiders may have been affected by this special ecology. Endosymbionts play critical roles for host adaptation and survival in secluded habitats, like the cave environment. Some cave-dwelling animals owe their life to chemolithoautotrophic bacteria (Engel et al., 2010; Sarbu et al., 1996) and many novel genera of bacteria have been reported (Morse et al., 2012; Paoletti et al., 2011). Here, we undertake a *Wolbachia* survey in cave spiders (*Telema* species) and discovered a new *Wolbachia* supergroup, R. Deduced from the single haplotype of *wsp* sequences, almost all of the *Telema* species we detect are infected by a single *Wolbachia* strain, which belongs to supergroup R (Fig. 1), except *T. cucurbitina* complex sp. Cave_num10. For *T. cucurbitina* complex sp. Cave_num10, we did not obtain any information for which supergroup of *Wolbachia* it harbors (see below). The reproductive phenotype caused by *Wolbachia* in *Telema* species is unknown, but female biased sex-ratio distortion has been observed in *Telema* species (unpublished data), suggesting that this new *Wolbachia* supergroup may induce male killing or parthenogenesis in *Telema* spiders.

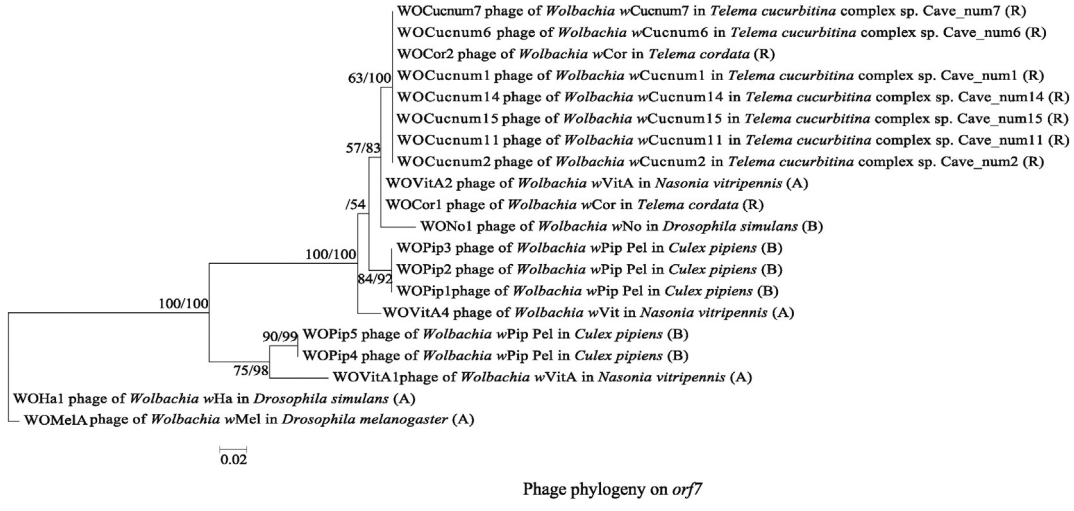

Fig. 2. Phage WO phylogeny based on *orf7*. The phage WO phylogeny was constructed based on Maximum Likelihood (ML) and Bayesian Inference (BI) of *orf7* sequences (123 bp) using the HKY model. ML bootstrap values based on 1000 replicates and Bayesian posterior probabilities are depicted as numbers beside each branch (the first number is ML value and the latter one is Bayesian probability). Only values larger than 50% are shown. The name for each sequence is given as the phage WO haplotype followed by *Wolbachia* strain, arthropod host, and a letter denoting the *Wolbachia* supergroup.

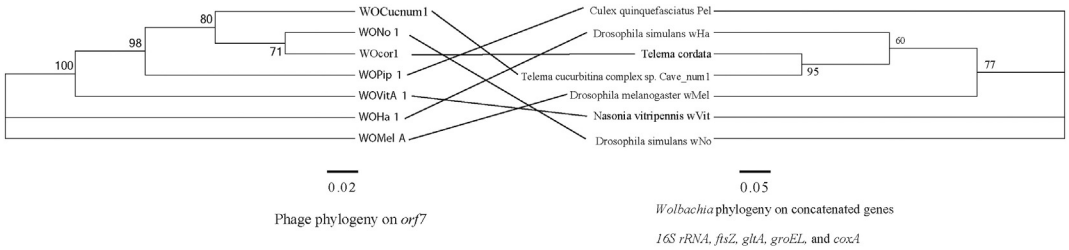

Fig. 3. Comparisons of phage WO and *Wolbachia* phylogenies. The phage WO phylogeny was constructed based on the Maximum Likelihood (ML) of *orf7* sequences (124 bp) using the HKY model. The *Wolbachia* phylogeny was constructed based on a supermatrix of five *Wolbachia* loci (*16S rRNA*, *coxA*, *ftsZ*, *gltA*, and *groEL*). ML bootstrap values on 1000 replicates are depicted, and only values larger than 50% are shown. For *Wolbachia*, the name of the host arthropod species is followed by the *Wolbachia* strain name if there is more than one *Wolbachia* strain infecting this species. For phage WO, the name is the phage WO haplotype. Each line between the two phylogeny trees connects the phage WO haplotype and *Wolbachia* strain that infecting the same arthropod species.

4.2. Wolbachia has more diversity than previously discovered

The increasing discovery of new *Wolbachia* supergroups (Augustinos et al., 2011; Bing et al., 2014; Glowska et al., 2015; Ros et al., 2009), including the new supergroup R reported here, has led us to rethink *Wolbachia* diversity and the relationships of different *Wolbachia* supergroups. To date, only *Wolbachia* supergroups A, B, C, and D have a robust relationship based on phylogenomics analyses (Comandatore et al., 2013). For other supergroups, because of limited data, the phylogenetic relationship is based on only single gene or part of the concatenated conserved genes. Therefore, the status and relative relationship of these supergroups need further confirmation (Bing et al., 2014; Glowska et al., 2015). In the present study in *Telema* species, both phylogenetic trees of a single gene or a supermatrix of five loci reveal a monophyletic pattern (Figs. 1 and S1), which supports the existence of a new *Wolbachia* supergroup R.

It is noteworthy that for a peculiar species, *T. cucurbitina* complex sp. Cave_num10, we do not obtain any amplification of the *Wolbachia* gene markers, *16S rRNA*, *fbpA*, *gatB*, *hcpA*, *ftsZ*, *gltA*, *groEL*, and *coxA* (Table 1), although the *Wolbachia* gene of *wsp* and phage WO gene *orf7* were positively detected (Table 1). Thus, we predict that this species may harbor a distinct *Wolbachia* strain or supergroup compared with the other *Telema* species. Many examples of certain genera or species harboring distinct *Wolbachia* lineages have been reported, e.g. in *Syringophilopsis turdi* (coinfected by supergroups F & P), *Torotrogla* (coinfected by supergroups F, P & Q) (Glowska et al., 2015), *Bemisia tabaci* (coinfected by supergroups B and O) (Bing et al., 2014), *Cinara* (coinfected by supergroups A, B, & M), *Toxoptera* (coinfected supergroups M & N) (Augustinos et al., 2011), and *Bryobia* (coinfected by supergroups B & K) (Ros et al., 2009).

4.3. Phage WO can horizontally transfer among distant Wolbachia supergroups

Phage WO was first sequenced in the year of 2000 (Masui et al., 2000). Since then, many more phage WO genomes have been reported along with *Wolbachia* genome sequences (Wang et al., 2013). There has been evidence that phage WO can transfer between different *Wolbachia* strains (Chafee et al., 2010; Gavotte et al., 2004) and different *Wolbachia* supergroups (Kent et al., 2011). However, all of these examples are from *Wolbachia* supergroups A and B. Mutualistic *Wolbachia* supergroups C and D have lost phage WO (Darby et al., 2012; Foster et al., 2005). To our knowledge, except in *Wolbachia* supergroups A–D and F, there have been no studies of phage WO in the other known *Wolbachia* supergroups. Here, we report that phage WO also exists at a high infection rate (100%) in *Wolbachia* supergroup R (Table 1), and phage WO can horizontally transfer between *Wolbachia* supergroups A and R (Fig. 1). This indicates that these two *Wolbachia* supergroups, though the relative relationship between is more distant than between supergroups A and B, may coinfect the same hosts in nature. These results also raise new questions. For example, does phage WO infect the other *Wolbachia* supergroups (E and H–Q)? If so, what is the evolutionary route of phage WO among these distinct *Wolbachia* supergroups? The answers to these questions will be determined in future studies based on more comprehensive survey studies.

5. Conclusion and outlook

We conducted a comprehensive detection of *Wolbachia* and phage WO in *Telema* cave spiders. Our findings have broadened the host spectrum of *Wolbachia* (detection of a novel supergroup) and phage WO (infecting the novel *Wolbachia* supergroup). Although *Telema* species live in cave environments are confined to a small geographic area, and have little communication with the external biota (Zhang and Li, 2013), phage WO lateral transfer between distinct *Wolbachia* supergroups has occurred. This indicates that *Telema* species and their *Wolbachia* endosymbionts may be a good model for the exploration of horizontal transfer of phage WO.

Author contributions

GHW, conception and design, acquisition of data, analysis and interpretation of data, drafting or revising the article; DWH, conception and design; JHX, analysis and interpretation of data, and drafting of the article; LYJ, analysis data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.03.015.

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