"*Candidatus* Mesochlamydia elodeae" (Chlamydiae: *Parachlamydiaceae*), a novel chlamydia parasite of free-living amoebae

Daniele Corsaro · Karl-Dieter Müller · Jost Wingender · Rolf Michel

Abstract *Vannella* sp. isolated from waterweed *Elodea* sp. was found infected by a chlamydia-like organism. This organism behaves like a parasite, causing the death through burst of its host. Once the vannellae degenerated, the parasite was successfully kept in laboratory within a *Saccamoeba* sp. isolated from the same waterweed sample, which revealed *in fine* through electron microscopy to harbor two bacterial endosymbionts: the chlamydial parasite we introduce and another endosymbiont initially and naturally present in the host. Herein, we provide molecular-based identification of both the amoeba host and its two endosymbionts, with special focus on the chlamydia parasite. High sequence similarity values of the 18S rDNA permitted to assign the amoeba to the species *Saccamoeba lacustris* (Amoebozoa, Tubulinea). The bacterial endosymbiont naturally harbored by the host belonged to *Sphingomonas koreensis* (Alpha-Proteobacteria). The chlamydial parasite showed a strict specificity for *Saccamoeba* spp., being unable to infect a variety of other amoebae, including *Acanthamoeba*, and it was itself infected by a bacteriophage. Sequence similarity values of the 16S rDNA and phylogenetic analysis indicated that this strain is a new member of the family *Parachlamydiaceae*, for which we propose the name "*Candidatus* Mesochlamydia elodeae."

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Introduction

Chlamydiae constitute a large group of intracellular parasites of eukaryotes, infecting amoebae and some invertebrates and vertebrates, including humans (Corsaro and Venditti 2004; Corsaro and Greub 2006). Most endosymbionts of amoebae form the monophyletic family *Parachlamydiaceae*; the first recognized members, recovered within environmental and clinical isolates of *Acanthamoeba* (Fritsche et al. 1993; Michel et al. 1992, 1994), include *Parachlamydia acanthamoebae*, *Protochlamydia amoebophila*, and other unnamed strains (Amann et al. 1997; Collingro et al. 2005; Fritsche et al. 2000).

Acanthamoeba spp. (Amoebozoa, Centramoebida) are na-ked free-living amoebae widespread in many environments and are able to cause by themselves diseases in vertebrates, forming the heterogeneous functional group of amphizoic amoebae (Visvesvara et al. 2007). Many *Acanthamoeba* strains harbor endosymbionts and may be vehicle for various pathogens. Due to their medical importance as well as their easy management in laboratory in terms of recovery,

maintenance, and propagation, *Acanthamoeba* spp. are mostly studied as natural and experimental hosts for emerging pathogens, including chlamydiae (Horn and Wagner 2004; Corsaro and Greub 2006). A wider host range for all chlamydiae was suggested from early environmental molecular surveys show-ing huge phylotype diversity (Horn and Wagner 2001; Corsaro et al. 2002) as well as from experimental studies using a large variety of amoeba species (Michel et al. 2004, 2005) and from mixed cocultures (Corsaro and Venditti 2009). This was further confirmed by finding chlamydiae in "unusu-al" hosts (e.g., Thao et al. 2003; Corsaro et al. 2007; Israelsson 2008). Many strains and species of *Parachlamydiaceae* how-ever have been recovered from *Acanthamoeba*, either as natural endosymbionts or by coculture (e.g., Schmitz-Esser et al. 2008; Corsaro et al. 2009; Matsuo et al. 2010). Nevertheless, *Neochlamydia hartmannellae* inhabits *Hartmannella* (*Vermamoeba*) *vermiformis* (Amoebozoa, Echinamoebida) but failed to infect *Acanthamoeba* (Horn et al. 2000), and *Protochlamydia naegleriophila* resides naturally in *Naegleria* spp. (Excavata, Heterolobosea) and grows easily within *Acanthamoeba* and several other amoebae (Michel et al. 2000). By contrast, *Metachlamydia lacustris* parasitizes naturally nonamphizoic *Saccamoeba* spp. (Amoebozoa, Euamoebida) and fails to infect amphizoic amoebae like *Acanthamoeba*, *Vermamoeba*, and *Naegleria* (Michel et al. 2006; Corsaro et al. 2010).

Recently, Michel et al. (2010) isolated *Vannella* sp. heavily infected by intracellular bacterial parasites from the waterweed *Elodea* sp. (Angiospermae, Alismatales, Hydrocharitaceae), a basal monocot generally used as aquarium vegetation. Vannellae rapidly degenerated, but a parasite was preserved by transferring it to a *Saccamoeba* sp. also isolated from the same *Elodea* sample. Electron microscopy analysis revealed that the new amoeba host harbored finally two different types of endosymbionts. The endosymbiont presumably responsible for the death of the vannellae, called KV, exhibited a chlamydia-like morphology and was infected by a bacteriophage (Michel et al. 2010).

This study focuses on the molecular phylogenetic analysis based on the small subunit rRNA genes of both the *Saccamoeba* host and the chlamydia-like endoparasite KV. Our results confirm amoeba to be a new strain of *Saccamoeba lacustris* and show that the strain KV belongs to a new genus-level lineage within the family *Parachlamydiaceae*, for which we propose the name "*Candidatus* Mesochlamydia elodeae".

Materials and methods

Samples

Original infected amoebae hosts, identified morphologically as *Vannella* sp., were isolated from leaves of commercial waterweed *Elodea* sp. (Angiospermae, Alismatales). From the same sample, some uninfected amoebae, identified morphologically as *Saccamoeba limax*, were also recovered and used to successfully propagate the endocytobiont after the decay of the original vannellid host (Michel et al. 2010). Morphological identification of amoebae was performed according to Page (1988).

Clonal subpopulations of infected and uninfected *Saccamoeba* (strain SL-elo) were maintained for over 1 year at room temperature on 1.5 % nonnutritive agar (NNA) covered with *Enterobacter cloacae* or *Escherichia coli*. To allow maintenance of the endosymbionts, infected amoebae were periodically transferred to fresh NNA plates containing uninfected amoebae as new hosts.

Amoeba coculture and host range

A preliminary amoeba host range for KV, including various *Saccamoeba* spp. (*Saccamoeba limax*, *Saccamoeba lucens*, *Saccamoeba lacustris*) and other Amoebozoa (e.g., *Acanthamoeba lenticulata*, *Sappinia* spp., *Thecamoeba* spp.) and Heterolobosea (e.g., *Naegleria*, *Tetramitus*), was studied previously (Michel et al. 2010). In this study, further *Acanthamoeba* (genotype T4) and *Vermamoeba* cocultures were performed, as previously described (Corsaro et al. 2009, 2010). Briefly, trophozoites were prepared as host cells in six-well microplates in Page's amoeba saline (PAS), inoculated with chlamydiae and incubated at room temperature in a humidified atmosphere in the dark. Five days postinfection, wells were screened by chlamydia-specific PCR (see below). *Saccamoeba* strain SL-elo was grown onto inactivated bacteria, thus inoculated with chlamydiae and incubated at room temperature. Amoebae were inspected daily at light microscope and screened for chlamydiae by PCR.

DNA amplification, sequencing, and phylogenetic analysis

Amoebae were harvested from the agar plates, suspended in PAS, and rinsed three times in PAS at 200×*g*. Infected amoebae were freeze-thawed, and further low-speed centrifugation steps were applied to separate amoebal cell debris from the cytoplasm containing the endosymbionts.

Whole DNA was extracted with the Wizard Genomic DNA kit (Promega) according to the manufacturer's recommendations. Amoebal 18S rRNA gene was amplified by using the primers 42F (5′-CTC AAR GAY TAA GCC ATG CA-3′) and 1498R (5′-CAC CTA CGG AAA CCT TGT TA-3′) (López-García et al. 2007) and 6F (5′-CCA GCT CYA AKA GCG TAT ATT-3′) and 9R (5′-GTT GAG TCR AAT TAA GCC GC-3′) (modified from Corsaro et al. 2009), in the reaction conditions of 5 min at 94 °C, followed by 35 cycles for 1 min at 94 °C, 1 min at 56 °C, and 2 min at

72 °C, with a final extension of 5 min at 72 °C. Chlamydial 16S rRNA gene was amplified by using the pan-chlamydia primers CF1 (5′-CGT GGATGA GGC ATG CRA GTC G-3′) and CR7 (5′-TAC CTT GTT ACG ACT TMA YCC YAG-3′) (Corsaro and Venditti 2009; Corsaro and Work 2012), under the reaction conditions of 5 min at 94 °C, followed by 35 cycles for 1 min at 94 °C, 1 min at 60 °C, and 1 min 30 s at 72 °C, with a final extension of 5 min at 72 °C. Bacterial 16S rDNAs from additional endosymbionts were amplified by using the eubacterial primers EBF (5′-AGA GTT TGA TCM TGG CTC AG-3′) and EBR (5′-ACG GCT ACC TTG TTA CGA CTT-3′) (Corsaro and Venditti), as well as the primers alpha-F19 (5′-CCT GGC TCA GAA CGA ACG-3′) and alpha-R1517 (5′-TGATCC AGC CGC AGG TCC-3′) specific for Alpha-Proteobacteria (Vannini et al. 2004). PCR con-ditions were 5 min at 94 °C, followed by 35 cycles for 1 min at 94 °C, 1 min at 51 °C or 56 °C, respectively, and 1 min 30 s at 72 °C, with a final extension of 5 min at 72 °C. A 600-bp 16S rDNA fragment was separately amplified with eubacterial primers 519f (5′-CAG CAG CCG CGG TAA TAC-3′) and 1100r (5′-GGG TTG CGC TCG TTG-3′) and cloned in *Escherichia coli* by using the TOPO TA Cloning System (Invitrogen). Six clones were randomly selected for sequencing.

Purified PCR products were sequenced with the same primer sets and a series of inner primers by using an automatic ABI DNA Sequencer (Applied Biosystems) with the BigDye Terminator Cycle Sequencing Kit. Sequences were edited by using BioEdit and analyzed through BLAST server to search for closest relatives. SSU rDNA sequences retrieved from GenBank were aligned by using MUSCLE.

Phylogenetic analyses were performed by applying distance (neighbor joining, NJ) and maximum parsimony (MP) with MEGA5 (Tamura et al. 2011), and maximum likelihood (ML, GTR, G+I:4 model) with TREEFINDER (Jobb et al. 2004), with 1,000 bootstraps.

Sequence similarity values were calculated with BioEdit. An overall sequence similarity matrix, including one member for each major species of all *Chlamydiae*, was calculated using all the common sites and excluding indels. Genetic relatedness of KV with *Parachlamydiaceae* was further analyzed by considering near full 16S rDNA sequences representing the various species and clades within this family, as defined by previous studies (Corsaro and Venditti 2006, 2009; Corsaro et al. 2010).

Electron microscopy

Methods for electron microscopy were reported previously (Michel et al. 2006, 2010). Briefly, infected *Saccamoeba* trophozoites were harvested from NNA plates, pelleted for 15 min at 200×*g*, fixed for 1 h in 3 % ice-cold glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, postfixed for 1 h in 1 % osmium tetroxide and 2 % uranyl acetate, dehydrated in alcohol, and embedded in Spurr resin. Sections were stained with 1 % lead citrate.

Fluorescence in situ hybridization

Harvesting of KV-infected amoebae from agar plate cultures, fixation, and dehydration of the cells were performed according to the procedure described by Grimm et al. (2001). Fluorescence in situ hybridization (FISH) was performed on KV-infected amoebae, using the oligonucleotide probe Chls-523, 5′-labeled with the fluorescent dye Cy3, specific for *Chlamydiales* as described in Poppert et al. (2002). The cells were viewed under an epifluorescence microscope (Carl Zeiss Microscopy GmbH, Germany) using Filter Set 20.

Results and discussion

The putative natural amoeba host *Saccamoeba* strain SL-elo

The strain SL-elo was isolated from the same *Elodea* sample, from which vannellid amoebae infected with the chlamydia KV originated, and was successfully used for its propagation (Michel et al. 2010). By considering both its origin and high susceptibility, this strain might be a natural host for KV. We thus consider this *Saccamoeba* species as a putative natural host.

Morphologically, SL-elo was very similar to *Saccamoeba limax* sensu Page and it was described as belonging to this species in the first publication (Michel et al. 2010). No cyst formation was observed for over 1 year of cultivation onto NNA (Michel et al. 2010; this study). On the basis of near full 18S rDNA sequence, SL-elo (GenBank account no. JN112797) showed 99.5 % similarities with *Saccamoeba lacustris* strain SL2 (CCAP 1572/4) (Corsaro et al. 2010) and 95.7 % with *Saccamoeba limax* strain NTSHR, isolated from decomposing gills of aquarium fish (Dyková et al. 2008), followed by *Glaeseria mira* with 86.4 %. Similarity value with the strain F-13 ATCC 30942, designed as *Saccamoeba limax*, was only of 77.5 %. In phylogenetic reconstruction (Fig. 1), our *Saccamoeba* strain emerged within the newly recognized *Saccamoeba* lineage represented by strains SL2 and NTSHR, as sister group to *Glaeseria mira* within the Hartmannellidae (Euamoebida, Tubulinea) (Smirnov et al. 2011). We assigned thus the strain SL-elo to the species *Saccamoeba lacustris*. It has been deposited at the Culture Collection of Algae and Protozoa (CCAP 1572/6). The *Saccamoeba*/*Glaeseria* is sister to the *Copromyxa* lineage, which includes the *Hartmannella cantabrigensis*/*Copromyxa* clade sensu Smirnov et al. (2011), and the strain 4730 MK-2011

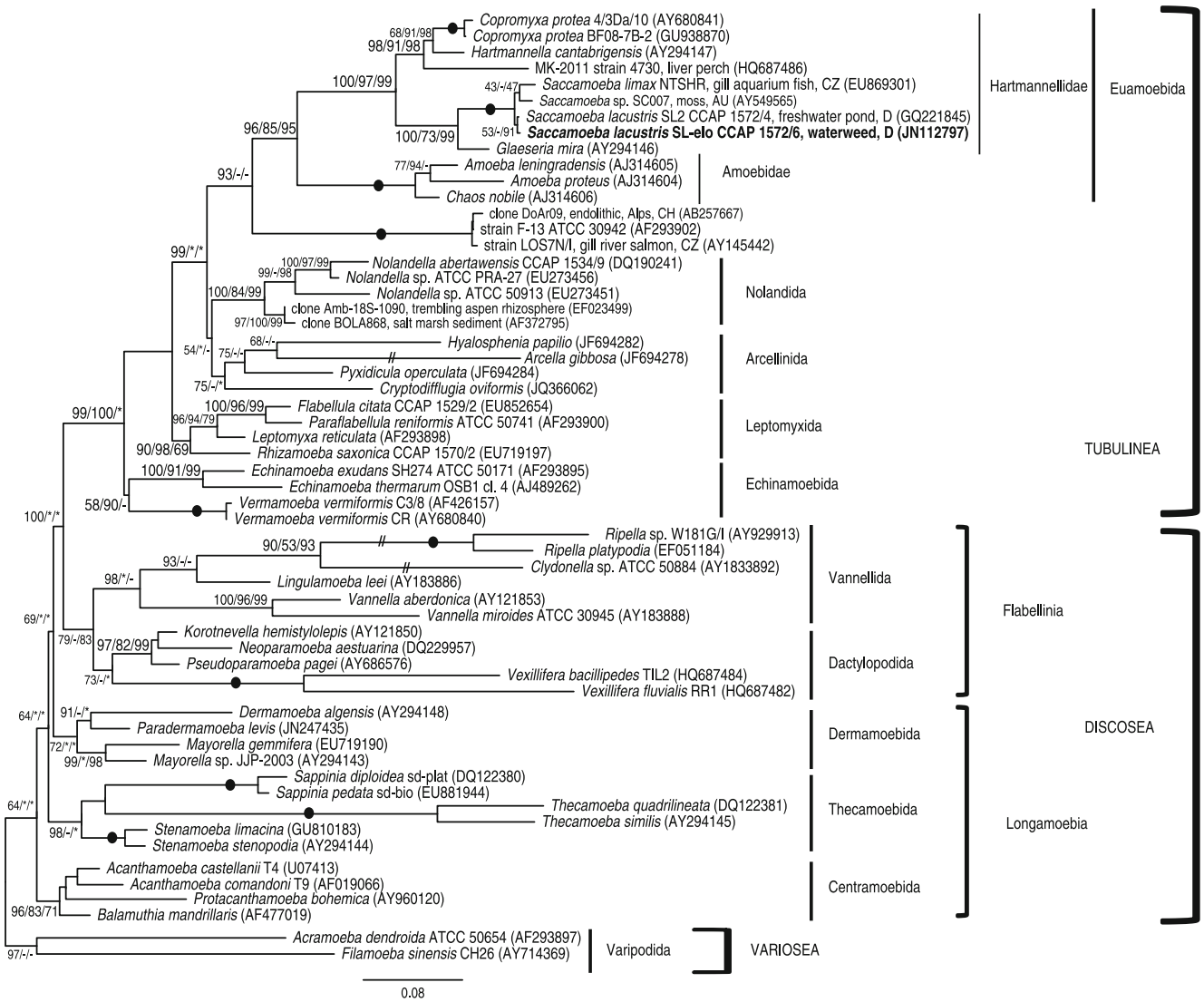

Fig. 1 Maximum likelihood 18S rDNA tree of Lobosa (Tubulinea + Discosea), showing the major inner groups, and the position of the recovered *Saccamoeba lacustris* strain SL-elo (*in bold*) in the family Hartmannellidae. *Acramoeba dendroida* and *Filamoeba sinensis* (Varipodida, Variosea) were used as outgroup. Bootstrap values after 1,000 replicates for ML/NJ/MP were indicated at nodes. *Filled circle*, node 100 % supported with all three methods; *asterisk*, node supported but BV <40 %; *hyphen*, node not supported. The *scale bar* represents substitution/site

(Dyková et al. 2011), early misidentified as *Vexillifera expectata*. We further confirmed that the strain F-13 ATCC 30942 is not a member of the genus *Saccamoeba*, emerging with other two closely related phylotypes in a distinct branch within the Tubulinea. We suggest to consider the strain NTSHR as a neotype for *Saccamoeba limax*.

The putative endosymbiont of *Saccamoeba*

Electron microscopy showed that a different type of endosymbiont was harbored in at least some subclonal populations of our *Saccamoeba* strain (Michel et al. 2010). The presence of endosymbionts was considered to be a typical feature of this amoeba genus by Page (1988). A unique 16S rDNA sequence was obtained from both uninfected and KV-infected *Saccamoeba* plates, suggesting that this putative symbiont was originally present within *Saccamoeba* and did not derive from vannellae along with KV. The sequence was obtained from uninfected amoebae with eubacterial primers by direct sequencing of PCR products and after cloning, indicating that the recovered sequence likely represents the dominant phylotype. The same sequence was obtained from KV-infected amoebae with primers specific for Alpha-Proteobacteria, to avoid coamplification of chlamydial DNA.

This putative endosymbiont belongs to the genus *Sphingomonas* (Alpha-Proteobacteria, Sphingomonadales), the obtained sequence (GenBank account no. JN112798)

showing 99.8 % similarity with the type strain JSS-26 of *Sphingomonas koreensis*, isolated from natural mineral wa-ter (Lee et al. 2001). Similarity value with the *Elodea* epiphyte *Sphingomonas elodea* (GenBank account no. AF503278), an important producer of exopolysaccharide known as gellan (Fialho et al. 2008), was of only 96.1 %, thus allowing to reasonably exclude a contamination. *Sphingomonas* spp. are widespread in the environment and have occasionally been isolated in *Acanthamoeba* cocul-tures (e.g., Evstigneeva et al. 2009). However, these bacteria are rod-shaped measuring 0.5×1.5–2-μm, while our amoe-bae showed at electron microscopy smaller irregularly shaped but mostly coccoid Gram-negative endosymbionts (Fig. 2). One possibility may be that strict endosymbiosis lifestyle has led to a change from rod to coccus shape for this strain. Interestingly, shorter (0.6–0.8 μm in length) *Sphingomonas* endosymbionts, matching 100 % with *S. koreensis* JSS-26 and our putative symbiont in partial 16S rDNA sequences, were found in the cytoplasm of the testate amoeba *Arcella rotundata* (Amoebozoa, Arcellinida) (Török et al. 2008); such shape change was supported by the find-ing of coccoid *Paenibacillus* as endosymbionts in the *Laccaria* mycelium (Bertaux et al. 2003). Since we did not explore more deeply this association in the present study, further research is now needed to confirm the real corre-spondence between molecular and microphotographic data, as well as to test the strict endosymbiotic hypothesis.

The chlamydial endoparasite KV

The chlamydial strain KV was unable to grow in amoeba coculture in microplates or NNA, using various amoebae as hosts, including both Amoebozoa (*Acanthamoeba*, *Flamella*, *Sappinia*) and Heterolobosea (*Naegleria*, *Tetramitus*, *Willaertia*). KV was able to grow only within strains of *Saccamoeba* spp. and it was thus kept within *Saccamoeba lacustris* strain SL-elo by periodic passages onto fresh troph-ozoites for over 1 year (Michel et al. 2010; this study). Presence of KV in *Saccamoeba* was documented by electron microscopy (see below) and FISH (Supplementary Fig. 1).

The near full-length 16S rDNA of KV was obtained from infected *Saccamoeba* cells by using PCR primers specific for chlamydiae. Overall, sequence similarities of the 16S rDNA from KV were 91.0 to 91.8 % with members of *Parachlamydiaceae*, 88.4–89.1 % with *Criblamydiaceae* and less than 89 % with members of the other chlamydial lineages (Supplementary Table 1). Sequence similarity values of >90 and >95 % have been proposed to include strains within the same family or genus, respectively (Everett et al. 1999; Corsaro et al. 2003). KV appeared thus as a new genus-level taxon within the family *Parachlamydiaceae*. To better infer genetic relatedness within *Parachlamydiaceae*, similarity values were calculated considering only members of this family (Supplementary Table 2). KV had a maximum similarity value of 91.87 % with *Metachlamydia lacustris*,

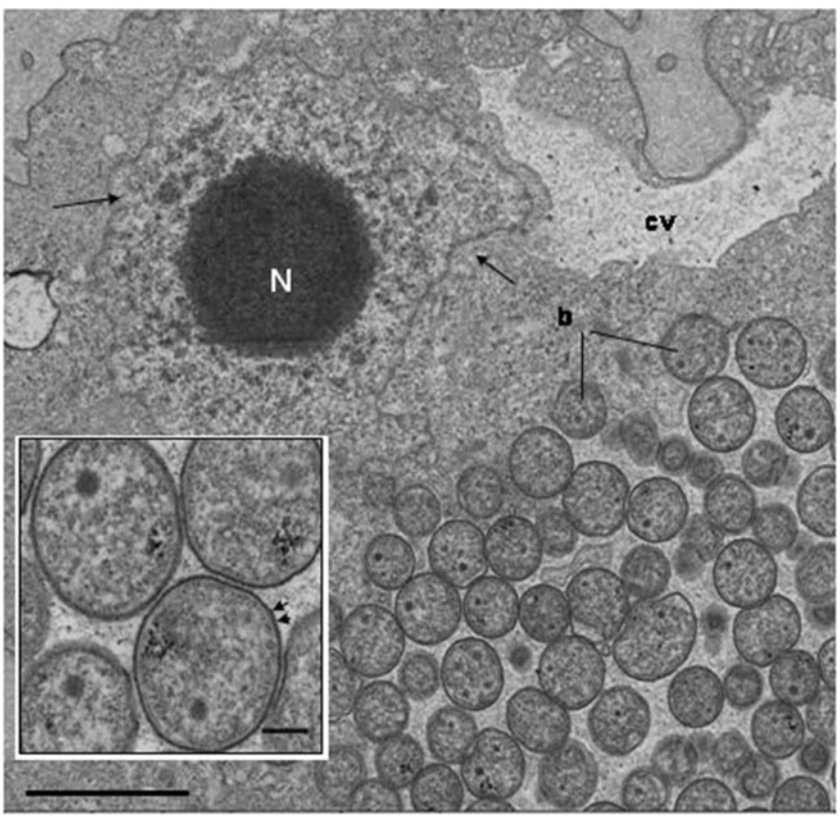

Fig. 2 Coccoid endosymbionts (*b*) within *Saccamoeba*, beneath the contractile vacuole (*cv*). Vesicular nucleus (*N*) typical of *Saccamoeba*. *Scale bar* 2 μm. *Inset*: Enlarged view of coccoid symbionts showing Gram-negative structure (*arrows*). *Scale bar* 200 nm. The figures are reproduced from a former article (Michel et al. 2010) with the kind permission of the publisher of "Endocytobiosis and Cell Research": http://zs. thulb.uni-jena.de/content/main/journals/ecb.xml?lang=en

followed by *Parachlamydia acanthamoebae* (mean value 91.7 %). Mean similarity values shared by KV with the other parachlamydiae were 88.1–91.5 %.

In molecular phylogenetic analyses based on 16S rRNA gene sequence (Fig. 3), *Parachlamydiaceae* was recognized as a holophyletic group with moderate to high bootstrap supports. *Criblamydiaceae* emerged as the sister group but with low support. Inner groups within *Parachlamydiaceae* were highly supported and corresponded to the previously identified clades (Corsaro and Venditti 2009). The strain KV emerged as a unique lineage within *Parachlamydiaceae*, h a v - ing a moderate ML support (70 %) for a relationship with *Metachlamydia*, corvenA4 group, and *Neochlamydia* lineage.

Typical chlamydial developmental cycle was documented for strain KV infecting *Saccamoeba* through electron micros-copy (Michel et al. 2010). Highly pleomorphic reticulate bodies (RBs), approximately 1 μm in diameter, reside mainly within a large cytoplasmic inclusion of the amoeba host, where they divide by binary fission (Fig. 4a, c). Elementary bodies (EBs), approximately 0.5 μm in diameter, rounded and edged, with a highly condensed central nucleoid, reside in smaller vacuoles containing one to three cells (Fig. 4b, c). Intermediate stages were not only mainly observable inside inclusions containing RB but also within small vesicles originated after the amoeba burst; 5 to 7 days postinfection, amoebae were completely filled and burst, releasing EB and small vesicular membranes containing chlamydiae at different stages (Fig. 4d). Some RBs contain both empty and filled hexagonal bacteriophages of about 55 nm in diameter (Fig. 5). Highly pleomorphic RB were observed in the related *Metachlamydia*, also infecting *Saccamoeba* (Michel et al. 2006; Corsaro et al. 2010), whereas other parachlamydial

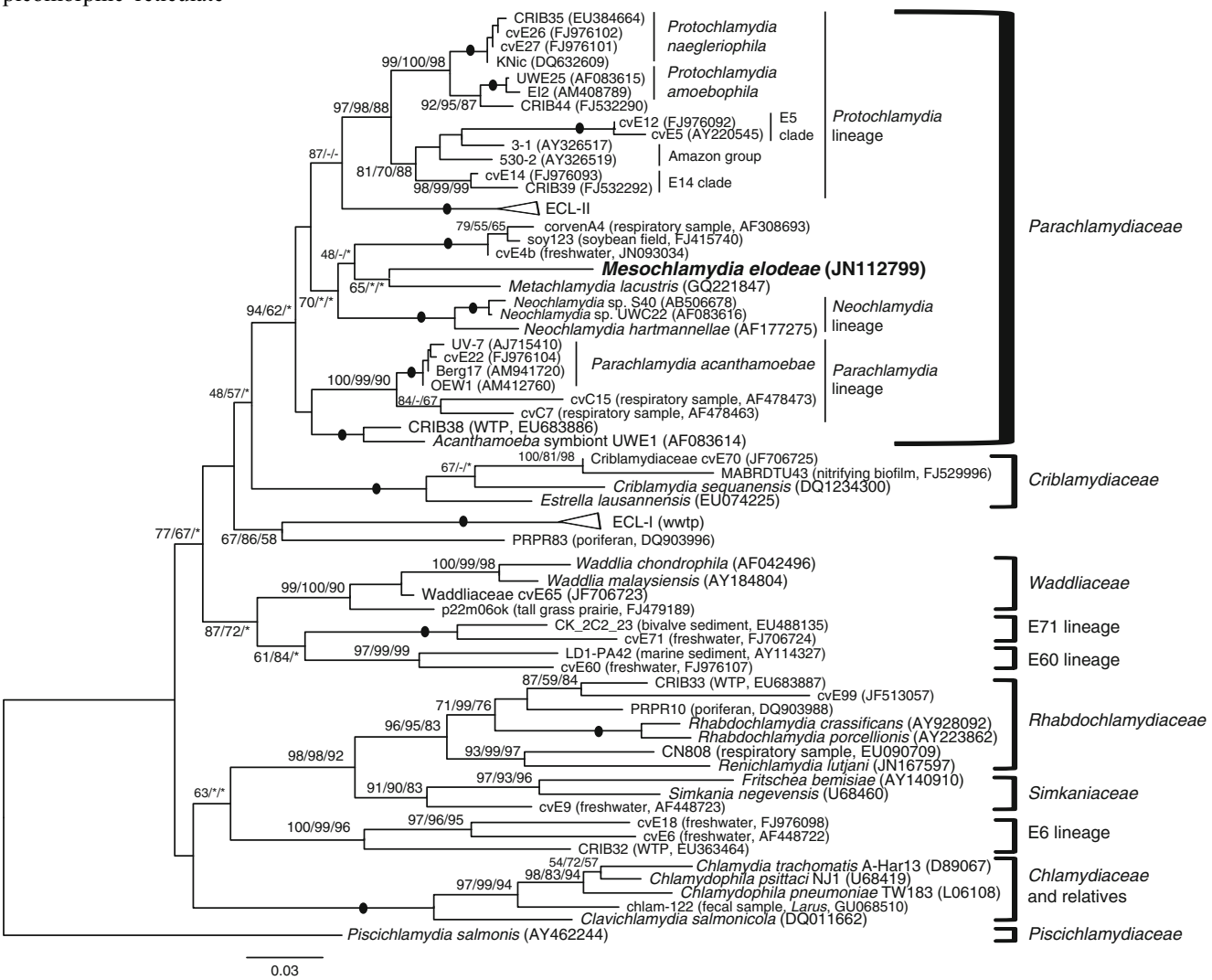

Fig. 3 Maximum likelihood 16S rDNA tree of *Chlamydiae*, showing the major lineages and sublineages, and the position of the recovered *Mesochlamydia elodeae* (*in bold*) within the *Parachlamydiaceae*. Bootstrap values after 1,000 replicates for ML/NJ/MP were indicated at nodes. *Filled circle*, node 100 % supported with all three methods; *asterisk*, node supported but BV <40 %; *hyphen*, node not supported. The *scale bar* represents substitution/site

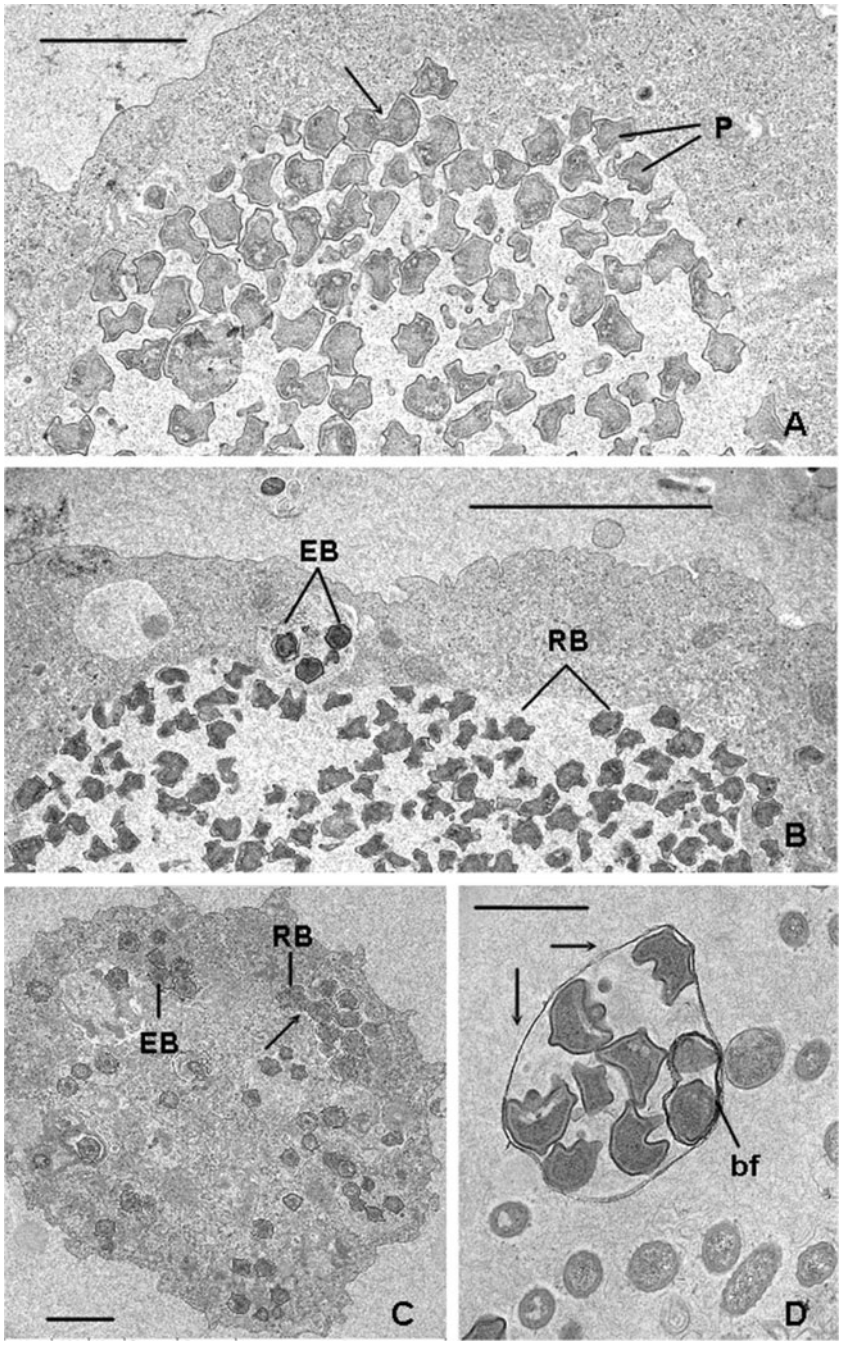

Fig. 4 *Saccamoeba* infected with *Mesochlamydia*. **a** Large inclusion in trophozoite showing irregular-shaped chlamydial parasites (*P*), mostly RBs, some in binary fission (*arrow*). *Scale bar* 2 μm. **b** Large inclusion with RB and a small inclusion with EB. *Scale bar* 5 μm. **c** EB with highly condensed central nucleoid and RB in binary fission (*arrow*). *Scale bar* 2 μm. **d** Vesicle released after amoeba burst containing *Mesochlamydia* cells at various stages, one of which in binary fission (*bf*). **c** was reproduced from a former article (Michel et al. 2010) with the kind permission of the publisher of "Endocytobiosis and Cell Research": http://zs.thulb.uni-jena.de/content/main/journals/ecb.xml?lang=en

taxa exhibit more rounded or wrinkled RB (e.g., Amann et al. 1997; Fritsche et al. 2000; Michel et al. 2000). Also, bacteriophages with similar morphology and size of 50–70 nm have been reported in other parachlamydiae strains infecting *Vermamoeba* (*H. vermiformis*) and *Naegleria* (Michel et al. 2001; Schmid et al. 2001), as well as in uncharacterized "chlamydia-like organisms" infecting bivalves (Harshbarger et al. 1977). These phages however appear different from the well-characterized but smaller, about 25 nm in diameter, *Chlamydiamicrovirus* (*Microviridae*: *Gokushovirinae*), infecting members of *Chlamydophila* spp. (*Chlamydiaceae*) (Everson et al. 2003).

It is to note that virome analyses in marine and freshwater biomes revealed unexpected high diversity and even dominant presence for chlamydiaviruses (Angly et al. 2006; Desnues et al. 2008; Rouxet al. 2012).

Description of "*Candidatus* Mesochlamydia elodeae"

Etymology: *Candidatus*, bacterial category including well-characterized but as yet uncultured organisms, among which are obligate intracellular bacteria. *Mesochlamydia* n. gen. Meso, Gr. prep. for middle, intermediate; Chlamydia, N.L. fem. n., a bacterial genus name; Mesochlamydia N.L. fem.

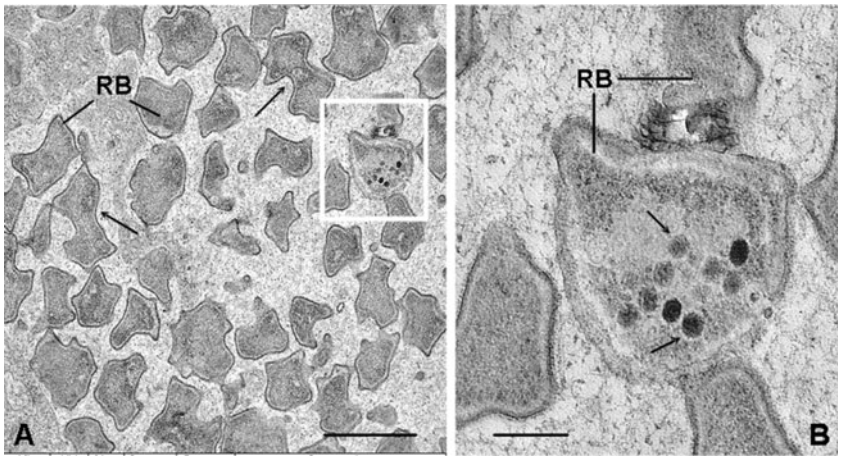

Fig. 5 *Mesochlamydia* infected with bacteriophages. **a** Pleomorphic RBs, some in binary fission (*arrows*), one showing phage particles (*inset*). *Scale bar* 1 μm. **b** Enlarged view of panel **a** showing both filled and empty bacterioph-ages within RB of *Mesochlamy-dia*. *Scale bar* 200 nm. The figures are reproduced from a former article (Michel et al. 2010) with the kind permission of the publisher of "Endocytobiosis and Cell Research": http://zs.thulb.uni-jena.de/content/main/journals/ecb.xml?lang=en

n., an intermediate genus-level lineage (between the other ones within the family *Parachlamydiaceae*). *Candidatus* Mesochlamydia elodeae n. sp., elodeae N.L. gen. sing. n., of *Elodea*, genus name of the waterweed from which the new chlamydia and the host amoebae were isolated.

Gram-negative, chlamydial developmental stages occurred within amoebae of the genus *Saccamoeba* (Amoebozoa, Tubulinea). Pleomorphic reticulate bodies, 0.8–1.1 μm in diameter, are generally inside a large cytoplasmic inclusion of the amoeba host, where they divide by binary fission. Some RB may contain hexagonal phages. Membrane-bounded vesicles containing single or small clusters of RB or intermediate bodies are also formed after the burst of the amoeba host. More rounded but edged elementary bodies, 0.5–0.6 μm in diameter, reside in single vacuoles or as small clusters in the cytoplasm.

The 16S rDNA (GenBank account no. JN112799) showed similarity values lower than 92 % with all the other members of *Parachlamydiaceae*, with a maximum value of 91.8 % with *Metachlamydia lacustris*. Phylogenetic analysis supported the emergence of strain KV as a unique genus-level lineage (sequence similarity with known members >90 and <95 %) within the family *Parachlamydiaceae*.

Natural host: Vannellid amoeba recovered from *Elodea* sp.; putative natural host: *Saccamoeba lacustris* strain SL-elo (CCAP 1572/6), recovered from *Elodea* sp. Observed behavior in sensitive amoebae is parasitic. Infection of other Amoebozoa and Heterolobosea was unsuccessful (Michel et al. 2010; this study).

Ecological considerations

Several strains of Amoebozoa and Heterolobosea were tested as host cells, but only strains of *Saccamoeba* spp. were found to successfully permit *Mesochlamydia elodeae* multiplication. Such a strict host preference for *Saccamoeba* spp. was recorded previously for *Metachlamydia lacustris*, which was unable to infect a variety of Amoebozoa and Heterolobosea under different growth conditions (Michel et al. 2006; Corsaro et al. 2010). Both chlamydiae seemed also to affect heterolobosean amoebae even if not able to multiply within them, *Metachlamydia* causing the formation of multinucleated cells in *Naegleria clarki* (Michel et al. 2006) and *Mesochlamydia* of empty cysts in *Tetramitus horticolus* (Michel et al. 2010).

An additional environmental *chlamydial* strain, cvE4b, recovered from a freshwater pond in France and kept in mixed protist coculture as described previously (Corsaro and Venditti 2009), also was unable to infect *Acanthamoeba* but rapidly killed *Saccamoeba*, suggesting that this amoeba is partially susceptible. This strain is closely related (98 % sequence similarity) to corvenA4, *a parachlamydia* recovered from a bronchoalveolar sample from a patient with pneumonia (Corsaro et al. 2001), emerging within the corvenA4 group sister to *Metachlamydia*/*Mesochlamydia* (Fig. 3).

These data suggest the existence of a possibly related group of parasites of saccamoebae or lobose amoebae in general, whose ecology is not based on *Acanthamoeba*. T o this putative group also belongs the *Neochlamydia* clade, with *Neochlamydia hartmannellae* specifically infecting *Vermamoeba* (*Hartmannella*) *vermiformis* but not *Acanthamoeba* (Horn et al. 2000).

Only few other partial *chlamydial* sequences showed some affinity with the putative group identified here. The 1,079-bp clone SOY123, from a soybean field, showed 99 % similarity with corvenA4, 92.9 % with *Metachlamydia*, and 92 % with *Mesochlamydia*. The 770-bp clones DDC2W1u16 (GenBank account no. EU634866), from showerhead biofilms, and BDC1_E12 (GenBank account no. AY689535), from pristine mountain stream sediment, shared 93.2–93.4 % similarities with *Mesochlamydia*, 94.5–94.9 % with *Metachlamydia*, and 93.9–96.7 % with corvenA4. Data on possible hosts for these phylotypes are lacking, as well as for their possible implication in human or animal infections. In this latter context, various recent studies employed the detection of either very small 16S

rDNA portions (<300 bp) and/or of highly species-specific PCR (e.g., qPCR) to recover novel chlamydiae from clinical samples. While increasing sensitivity and specificity in particular situations, these approaches however also underestimate the real incidence of these organisms for which, by definition, data are lacking.

Among other chlamydial groups, it is to note that *Waddlia chondrophila* (*Waddliaceae*) is able to infect a variety of vertebrate cell lines, but the infection potential of *Waddlia* for various amoebal strains was increased only after an adaptation to growth within *Hartmannella* (=*Vermamoeba*) which was highly susceptible from the start (Michel et al. 2004). Furthermore, several strains of various chlamydial lineages are unable to grow in *Acanthamoeba* (Corsaro and Venditti 2009). Two main chlamydiae infecting fish gills, *Piscichlamydia* and *Clavichlamydia*, failed to infect *Acanthamoeba* in vitro (Corsaro and Karslen, unpublished data), but it is possible that other protists and/or invertebrates can play the role of intermediate hosts. In fact, a large variety of amoebae other than *Acanthamoeba* may be recovered from both gills and internal organs of various fishes (Dyková and Lom 2004). Interestingly, recent studies strongly suggest environmental niche(s) for gills chlamydiae (Schmidt-Posthaus et al. 2012) and allowed to identify chlamydiae in internal organs of fish (Corsaro and Work 2012). Thus, the use of only *Acanthamoeba* as amoeba host for coculture and/or the search for natural endosymbionts/endoparasites present only in *Acanthamoeba* strains lead very likely to greatly underestimate both the real biodiversity and the possible host range of *Chlamydiae*.

Acknowledgments We thank Elke Schneider and Liane Junglas from the Electron Microscopy Department for the excellent technical assistance (Head of the department: Dr. Bärbel Hauröder). DC was partially supported by Novartis Foundation.

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