Molecular characterization of *Cardinium, Rickettsia, Spiroplasma* and *Wolbachia* in mite species from citrus orchards

Tatiana Pina[1,2], Beatriz Sabater-Muñoz[3,4], Marc Cabedo-López[1], Joaquín Cruz-Miralles[1], Josep A. Jaques[1], Mónica A. Hurtado-Ruiz[1*]

[1] Departament de Ciències Agràries i del Medi Natural, Universitat Jaume I (UJI), Campus del Riu Sec, 12071 Castelló de la Plana, Spain.

[2] Departament de Didàctica de les Ciències Experimentals i Socials, Universitat de Valencia, Avda. Tarongers, 46022 Valencia, Spain.

[3] Smurfit Institute of Genetics, Trinity College Dublin, University of Dublin. College Green, Dublin2 Dublin, Ireland.

[4] Integrative and Systems biology group, Dpt. Molecular mechanisms of stress in plants, Institute for Plant Molecular and Cell Biology (IBMCP) from the Spanish National Research Council (CSIC)- Polytechnic University of Valencia (UPV), Ingeniero Fausto Elio, 46022 Valencia, Spain.

Running title: Mite microbiota characterization in citrus

*Corresponding author:
Dr. M.A. Hurtado
Departament de Ciències Agràries i del Medi Natural
Universitat Jaume I
Campus del Riu Sec;
12071 Castelló de la Plana (Spain)
Tel: +34 963424000
Fax: +34 963424001
E-mail: mhurtado@uji.es

ORCID:
TP: 0000-0002-0374-4751
BSM: 0000-0002-0301-215X
JAJ: 0000-0003-1353-1727
MAHR: 0000-0003-2678-941X

Abstract

Tetranychidae spider mites are considered key citrus pests in some production areas, outstanding *Tetranychus urticae* Koch. Over the past decades, pesticide overuse seems to have promoted *T. urticae* population selection in citrus orchards. However, the microbiota has also been pointed out as a plausible explanation for population structure or the plant host specialisation observed in several arthropod species.

In this work, we have determined the incidence of *Cardinium, Rickettsia, Spiroplasma* and *Wolbachia* as representative of major distorter bacteria genera in *Aplonobia histricina* (Berlese), *Eutetranychus banksi* (McGregor), *E. orientalis* (Klein), *Panonychus citri* (McGregor), *T. evansi* (Baker and Pritchard), *T. turkestani* (Ugarov and Nikolskii), and *T. urticae* populations from Spanish citrus orchards.

Only *Wolbachia* was detected by PCR. The multilocus alignment approach and phylogenetic inference indicated that all detected *Wolbachia* belong to supergroup B. The deep analysis of each 16S rDNA, *ftsZ* and *wsp* gene sequences allowed identifying several phylogenetically different *Wolbachia* sequences. It probably indicates the presence of several different races or strains, all of them belonging to supergroup B. Whereas the *wsp* sequence typing analysis unveiled the presence of the two already-identified alleles (61 and 370), and allowed to contribute with five new alleles, supporting the presence of different but related B-races in the studied mite populations. The results are discussed and related to *T. urticae* population structure, previously observed in Spanish citrus orchards.

Keywords: 16S rDNA, multilocus alignment approach, Phylogeny, supergroup B, Tetranychidae, *Wolbachia*.

Introduction

Spider mites of the Tetranychidae family comprise more than 1300 phytophagous species, out of which about 10% are considered agricultural pests and approximately 10 are key pests of economically important crops (Migeon and Dorkeld 2019). *Tetranychus urticae* Koch is the most widespread mite, considered one of the citrus key pests, together with the Mediterranean medfly *Ceratitis capitata* Wiedemann and the diaspidid scale *Aonidiella aurantii* Maskell (Jaques et al. 2015). Phytoseiidae mites — either naturally present in the tree canopy or/and ground cover or introduced — are the natural enemies providing the biological control of these Tetranychidae, which in IPM can be complemented with a rational application of pesticides (Jacas et al. 2010). However, due to past abuse of pesticides, *T. urticae* populations in Mediterranean citrus orchards have shown a genetic structuration, which could be attributed to pesticide-driven selection (Pascual-Ruiz et al. 2014). Besides, other studies indicated the presence of selective mating forces or maternal factors that link *T. urticae* populations' genetic structure to plant host species, which could explain the genetic structuration observed, remains unsolved in citrus mites of Spain (Marinosci et al. 2015; Aguilar-Fenollosa et al. 2016; Sato et al. 2016).

By the mid-1960s, bacterial and yeast symbionts of arthropods and nematodes were highlighted as maternal factors affecting the ecology, evolution and reproductive biology of their hosts (Buchner 1965). Over the past two decades, this microbiota has become the focus of numerous studies, going from an ecological to a genomic perspective. More recently, the outcomes of these studies are being devised as a new form of biological control, by inducing reproductive barriers with the natural populations mediated by bacterial species (Zabalou et al. 2004; Atyame et al. 2011; Zhou and Li 2016). For example, cytoplasmic incompatibility (CI), a reproductive modification caused by some bacteria, can be used as a population suppression strategy, analogous to the sterile insect technique (SIT) that reduces or eliminates the population, or/and as population replacement, using the bacteria as a vehicle to drive desired phenotypes into natural populations (Brelsfoard and Dobson 2009). *Cardinium*, *Rickettsia*, *Spiroplasma* and *Wolbachia* are the representative genera of these bacterial distorters that infect many arthropod species (Jeyaprakash and Hoy 2000; Zchori-Fein and Perlman 2004; Engelstadter and Hurst 2009; Duron and Hurst 2013). *Cardinium* encompasses a bacterial genus of Bacteroidetes that induces reproductive alterations in its hosts such as cytoplasmic incompatibility, parthenogenesis and feminisation (Zchori-Fein and Perlman

2004; Zchori-Fein et al. 2004; Gotoh et al. 2007a; Zhu et al. 2012). *Rickettsia* and *Wolbachia* genera belong to Rickettsiales (within Alpha-Proteobacteria), forming two isolated clades that also induce reproductive alterations (as male feminisation, thelytokous parthenogenesis, cytoplasmic incompatibility and male death) and have also been related to pesticide resistance development (Werren 1997; Stouthamer et al. 1999; Stevens et al. 2001; Perlman et al. 2006; Hosokawa et al. 2010; Liu and Guo 2019). *Spiroplasma* belongs to the Mollicutes (within Firmicutes) and is also involved in the protection of its host against biotic and abiotic stresses (Bolanos et al. 2015; Heyworth and Ferrari 2015; Frago et al. 2017; Guidolin et al. 2018). Recent estimations of arthropod bacterial infestation reached up to 13% for *Cardinium,* 24% for *Rickettsia,* 5–10% for *Spiroplasma* and to 52% for *Wolbachia* (Duron et al. 2008; Weinert et al. 2015; Mathé-Hubert et al. 2019).

All these four genera are transmitted mainly vertically, from mother to offspring, by transovarial infection of eggs. The horizontal transfer has also been reported, either plant-mediated or transmitted by some parasitoid species (Russell et al. 2003; Sintupachee et al. 2006; Oliver et al. 2010; Ahmed et al. 2015; Li et al. 2017). Due to their intracellular lifestyle (except for some *Spiroplasma* species), most of these bacteria cannot be grown outside their arthropod host and their identification depends on the application of molecular methods. While bacterial species' identification relies on the positive amplification with species-specific primers, located mainly in the multicopy 16S rDNA locus, the *Wolbachia* incompatibility strain assignment is performed by multiple-loci sequence alignment analysis (MLSA) and phylogenetic inference against reference strains (Russell et al. 2003; Ros et al. 2009). To date, sixteen *Wolbachia* supergroups (named with letters from A to Q, with some recombination events) have been established based on these MLSA analyses (Lo et al. 2002, 2007; Bordenstein and Rosengaus 2005; Ros et al. 2009; Augustinos et al. 2011; Pascar and Chandler 2018).

As indicated previously, some of these bacterial species are involved in cytoplasmic incompatibility (being able to modulate population genetic structure), pesticide resistance and biotic/abiotic stress resistance (water and temperature). Therefore, the determination of their presence in the natural populations of Tetranychidae is important to ascertain how they may affect the host population structure.

In this work, we studied the incidence and frequency of infection of *Cardinium*, *Rickettsia*, *Spiroplasma* and *Wolbachia* in different Spanish populations of citrus mites of economic importance, focusing mainly on *T. urticae*. The other mite species studied

were *Panonychus citri* (McGregor), *Aplonobia histricina* (Berlese), *Eutetranychus banksi* (McGregor), *Eutetranychus orientalis* (Klein), *Tetranychus evansi* Baker and Pritchard, *Tetranychus turkestani* Ugarov and Nikolski and Tarsonemidae *Poliphagotarsonemus latus* (Banks).

Material and methods

Specimens' collection

Table 1 lists the mites (mainly Tetranychidae) collected mainly from Spanish citrus orchards or from laboratory rearing colonies, and the other insect species used as positive controls for PCR. The number of specimens and/or per population are also included in Table 1.

DNA extraction and verification

Total DNA was extracted from isolated, ethanol-washed specimens following a modified 'salting out' protocol (Pérez-Sayas et al. 2015). Briefly, each surface-disinfected specimen was air-dried, isolated in a 1.5-ml Eppendorf tube and crushed in TNS + Prot-K solution at 60˚C; proteins were precipitated with 5 M NaCl by centrifugation and the nucleic acid fraction was precipitated with 2-propanol. The extracted DNA from non-Acari specimens was quantified with Nanodrop 2000® (Thermo Sci., Wilmington, DE, USA). Whereas, the Acari specimen's DNA extractions were subjected to PCR with 18SrDNA primers (see Table 2) to ascertain the presence of DNA, as previously done with minute specimens (Pérez-Sayas et al. 2015).

Cardinium, Rickettsia, Spiroplasma and *Wolbachia* diagnostic PCR

The incidence of each bacterial symbiont was determined by positive PCR reactions with specific primers (listed in Table 2), targeting the 16S rDNA in each specimen collected. Due to the limiting factor of Acari source DNA, a secondary specific amplification was devised over a first (primary) amplification of whole 16S rDNA fragment, using the universal primers listed in Table 2, as devised for other insect-bacteria groups (van Ham et al. 1997; Russell et al. 2003). The primary PCR was performed using 1 μl of DNA extraction, whereas the specific secondary and diagnostic PCR was performed with 1–2 μl of the primary PCR. Amplification conditions varied slightly between bacterial species (see Table 2 for reaction volume, magnesium concentration and annealing temperatures),

using 1 U of FIREPol® polymerase (FIREPol®, Solis BioDyne, Tartu, Estonia) with the appropriate 1x buffer, with 0.2 mM dNTPs and 0.4 mM of each primer. Amplification was performed in a Bio-Rad thermal cycler (Thermal Cycler Bio-Rad C1000™, Foster City, CA, USA) under the following amplification conditions: a first denaturing step at 92–95 °C for 2–5 min, followed by 30–40 cycles of 92–95 °C for 30 sec, 52–58 °C for 30 sec and 72 °C for 30–60 sec, with a final extension at 72 °C for 5 min (see S.I.). For each amplification run, at least one negative control (ultrapure water added instead of DNA sample) and one positive specimen (of the species listed in Table 1; at least one per symbiont species to be determined) were included to ascertain the false positives (either due to contaminated reagents or environmental contamination) and negatives (due to failure of amplification or low DNA concentration), respectively. Amplification was verified by agarose gel (2% low EEO DA Agarose, Pronadisa, Sumilab SL, Madrid, Spain) electrophoresis in 1x TAE, stained with GelRed (Biotium, Hayward, CA, USA). Single, expected-size PCR fragments were considered positive when matching the size of the positive controls. Each specimen was considered harbouring *Cardinium*, *Rickettsia*, *Spiroplasma* or *Wolbachia*, when at least two PCR reactions give positive results of the three performed.

Positive PCR fragments were independently purified with Illustra™ ExoStar™ (GE Healthcare Life Sciences, Buckinghamshire, UK) following the manufacturer's recommendations. Bidirectional Sanger sequencing using Bigdye™ terminator v3.1 cycle sequencing kit (Thermo Fisher Sci., Vilnius, Lithuania) with each amplification primer was performed at the Sequencing service of the University of Valencia (Servei Central de Suport a la Investigació Experimental [SCSIE], Universitat de València, Spain), following the manufacturer's instructions. Reactions were run in an ABI 3730XL DNA analyser (Thermo Fisher Sci, Carlsbad, CA, USA) following the manufacturer's instructions.

Wolbachia wsp and *ftsZ* amplification and sequencing

To assign *Wolbachia* into the established supergroups, we used the MLSA approach by amplifying and sequencing the genes corresponding to cell division protein FtsZ (*ftsZ*) and the *Wolbachia* surface protein (*wsp*), in addition to the 16S rDNA described above (Braig et al. 1998; Zhou et al. 1998; Lo et al. 2002; Casiraghi et al. 2005; Baldo et al. 2006). PCRs were conducted independently using 1–2 μl of undiluted specimen DNAs with primers and conditions, as listed in Table 2, using 1 U of FIREPol® polymerase with

0.2 mM dNTPs and 0.4 mM of each primer for the 16S rDNA amplification. Amplifications were performed in a Bio-Rad thermal cycler with the following amplification conditions: a first denaturing step at 94–95 °C for 2–5 min, followed by 36–40 cycles of 94–95 °C for 30 sec, 54–55 °C for 45–60 sec, and 72 °C for 60–90 sec, with a final extension at 72 °C for 5 min (see S.I.). Similarly, positive (other arthropods specimens harbouring known types of *Wolbachia* and/or *Wolbachia* positive *T. urticae* samples) and negative (DNA-free PCR mixture) controls were included in each amplification run. Positive PCR fragments were purified as described above and sequenced bidirectionally with amplification primers, at the same SCSIE sequencing service.

Sequence analysis

The consensus sequence for each PCR product was obtained using the programme STADEN Package (Staden 1996). Consensus sequences were blasted against the non-redundant database to confirm fragment identity prior to alignment construction (BLAST; Altschul et al. 1997).

16S ribosomal DNA, *ftsZ* and *wsp* obtained consensus sequences and those retrieved from databases were independently aligned using CLUSTALW (as in MEGA X; Kumar et al. 2018) (for 16S rDNA) or with GENEDOC (Nicholas and Nicholas 1994–98). In GENEDOC, we used Blosum62 score table for coding regions *ftsZ* and *wsp*, whereas, for 16S rDNA, we used PAM 65 score table, setting alignment cost at 20 for constant length, 8 for gap opening and 4 for gap extension (for *ftsZ* and *wsp*, alignment was performed with translated sequences, re-gapping the nucleotide alignments). Moreover, 16S rDNA, *ftsZ* and *wsp* consensus sequences were concatenated in a single FASTA file previously to perform the Multilocus sequence alignment (MLSA). Outgroups were retrieved from the databases and sequences corresponding to the same species were concatenated in the same order as the MLSA (S.I. Table S1).

The *wsp* sequences were assigned to the corresponding allelic profile by comparing the four hypervariable regions (HVRs) against the *Wolbachia wsp* Multilocus sequence typing (MLST) database (https://pubmlst.org/wolbachia/ [last accessed 10/March/2020]; Baldo et al. 2005). Novel allele sequences were submitted to the database curators for their inclusion as new alleles after they registered as new sequences in NCBI.

Gene tree inference was conducted in MEGA X, after determining the best-fit evolutionary distance model (GTR) for each gene alignment and for the MLSA, as

implemented in MEGA X. Bayesian phylogenies were obtained using a Markov Chain Monte Carlo (MCMC) method implemented in BEAST v1.10.4 programme (Suchard et al. 2018). BEAST output was analysed using TRACER v1.7.1, applying values of more than 200 of the effective sample size (ESS) (Rambaut et al. 2018). A maximum clade credibility tree was generated after burning 10% samples with posterior probability limit > 0.5 using TreeAnnotator, as implemented in BEAST. Species phylogroups were defined by a posterior probability > 0.95 using referenced strains, known to belong to these groups. The final trees were visualised with FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/ [last accessed 10/March/2020]).

Data availability

All new sequences have been deposited in GenBank from MN123012 to MN123230 for 16S rDNA, MN187577 to MN187703 for *wsp* gene region and MN187704 to MN187866 for *fts*Z gene region (see S.I. Table S1 for the complete list).

Results and Discussion

Incidence of *Cardinium*, *Rickettsia, Spiroplasma* and *Wolbachia* in mite populations

Cardinium, Rickettsia and *Spiroplasma* species' specific primer pairs gave negative results in all the mite species and populations tested, despite their amplification efficacy being positive with the corresponding arthropod control samples. All samples were tested for DNA presence, as routinely done with such minute specimens, by amplification of 18S rDNA (Pérez-Sayas et al. 2015). Only the 16S universal and *Wolbachia*-specific primers (either 16S rDNA, *ftsZ* or *wsp*) rendered positive results. *Wolbachia* was present in almost all the mite species and/or populations tested with a prevalence ranging from 10 to 100% (Fig. 1), as previously reported (Zug and Hammerstein 2012; Weinert et al. 2015; Zhu et al. 2018). The exception was *P. citri,* which showed a prevalence of 0–10%. This is the first time that *Wolbachia* is reported in this mite species (Zélé et al. 2018a, b; Zhu et al. 2018).

Other authors have detected double infections in *Tetranychus* species; two of them were included in our study, namely *T. urticae* and *T. evansi* (Enigl and Schausberger 2007; Weinert et al. 2009; Xie et al. 2016; Staudacher et al. 2017; Zélé et al. 2018a, b). These studies found that *T. trucantus* Ehara (Acari: Tetranychidae) showed the combinations *Wolbachia* and *Cardinium* or *Spiroplasma* and *Rickettsia,* whereas *T. evansi*, *T. ludeni* and *T. urticae* showed only the *Wolbachia* and *Cardinium* combinations

(Zhang et al. 2013, 2016; Zélé et al. 2018a). Indeed, the double infection *Wolbachia* and *Cardinium* (W + C) is the most common in Tetranychidae (Zélé et al. 2018a, b). All these studies used two specific primer pairs: one pair targeting the 16S rDNA in *Cardinium* and *Spiroplasma* and the second one targeting a species-specific gene (i.e., *gyrB* for *Cardinium*, *rpoB* for *Spiroplasma* and *gltA* for *Rickettsia*). As indicated, the diagnostic primers used, except *Cardinium* ones (see Table 2), differ from other mite working groups but render positive results with arthropod species used as positive controls (see Table 2 for references of each primer pair).

Our aim was to detect each bacterial species based on the same target gene to include all of them in a phylogenetic study to determine the presence of more than one strain, for which our primer selection based on previous works targeting the multicopy gene 16S rDNA. In previous studies, we have observed a population structure in *Tetranychus urticae* within Spanish populations (by microsatellite analyses), that may be attributed to different *Wolbachia* operational taxonomic units, as noticed in the present study (operational taxonomic units as described for 16S rDNA sequences diverging more than 3–5% as in microbiome analyses) (see next subheading) (Aguilar-Fenollosa et al. 2012; Pascual-Ruiz et al. 2014). However, as *Wolbachia* was the only bacterial reproductive distorter detected in our study, a deep analysis of the *Wolbachia* sequences obtained was required to clarify the situation.

Phylogeny of *Wolbachia* and strain identification

Wolbachia is a group of bacterial strains that can be assigned to supergroups following a Multilocus phylogeny approach, as indicated previously (Ros et al. 2009). The sequence of coding genes of the cell division protein FtsZ (*ftsZ* gene) or the *Wolbachia* surface protein (*wsp*) are routinely used for placement of *Wolbachia* strains into the established supergroups A to K (Zhou et al. 1998; Gotoh et al. 2003; Casiraghi et al. 2005; Baldo et al. 2007), whereas the 16S rDNA, is routinely used to determine bacterial species identity in microbiome studies.

Here, we have estimated the tree phylogeny of *Wolbachia* from several mite species with a Multilocus alignment (MLSA) of concatenated 16S rDNA, *wsp* and *ftsZ*, and then analysed each locus independently by different tree-reconstruction methods (Maximum Parsimony [MP], Maximum Likelihood [ML] and Bayesian). Using the MLSA approach, either by MP (MP was used to compare against precedent works as done by Zhang et al. 2013), ML or by Bayesian inference, almost all the mites new *Wolbachia* sequences

clustered within the supergroup B, except the ones from the Brazilian population of *T. evansi* (TeBr45 and TeBr70) that clustered either basal in the B group (Bayesian inference) or between A-, K-, C- *Wolbachia* supergroups (ML) (Fig. 2).

Due to the scarcity of DNA material obtained from these minute mites, it was impossible to obtain a sequencing grade *wsp* fragment for some samples, which limited the number of samples used for this MLSA to 90 individuals. Consequently, the power of MLSA to determine the presence of more than one *Wolbachia* strain in our samples was limited. When we analysed the MLSA solely composed by 16S rDNA + *ftsZ* fragments, we increased the figure to 121 newly concatenated sequences, despite that for the tree inference 100% identical sequences from the same mite population were removed to reduce the computing time (Fig. 3). Limiting data and samples reduced the resolution of the trees and improved deep branching in some cases; whereas in others, and due to positive selection detected in some *wsp* lineages, clustering of sequences belonging to same supergroups did not match previous works (Schulenburg et al. 2000; Ros et al. 2009). In this case (16S rDNA + *ftsZ*), all the *Tetranychidae* sequences clustered together within the supergroup B, not showing any structuration between the Brazilian population of *T. evansi* nor the already characterised as different members of the supergroup B (including in this last group all the B-*Wolbachia* from different insect species with different reproductive modes) (Ros et al. 2009, 2012).

Further, when each gene fragment was independently analysed, we could observe a supported differentiation that depends on the fragment type (coding or non-coding). Our limiting sequence (by the number of samples and available supergroups), *wsp*, gave different tree inferences (Fig. 4 and S.I. Fig. S1), keeping in both cases supergroups A, C and E as basal with high posterior probabilities or bootstrap values. While the B-supergroup was split into three clusters (called B1, B2 and B3; Fig. 4), the first two, B1 and B2, included many of the outgroup sequences. Some of them were linked but not completely isolating species with feminisation or thelytoky reproductive specialisations. Group B3 included many species (from outgroup) with identified cytoplasmic incompatibility, with all of our sequences (van Meer et al. 1999). Despite this, group B3 seemed to also show an internal split into three other groups with posterior probabilities higher than 0.96; the results did not find any relationship between *Wolbachia* taxonomic unit (B-sub-sub-strain; sequences that show high-sequence divergence, conforming differential taxonomic units) and host plant or mite populations, as previously found with microsatellites. Further, *ftsZ* phylogenies placed A-supergroup sequences in a basal

cluster to B-supergroup, which is subdivided into 3 subgroups (B1 to B3 in Fig. 5 and S.I. Fig. S2), on which again sequences of *T. evansi* from Brazil roots in the most basal subgroup (B1). The 16S rDNA phylogenies were most resoluteness, supporting the clustering of supergroups, as previously published (Fig. 6 and S.I. Fig. S3) (Gotoh et al. 2003, 2007b; Ros et al. 2009, 2012; Suh et al. 2015). With this marker, supergroup B was split into five subgroups (B1 to B5 in Fig. 6), with *T. evansi* Brazil population sequences mostly concentrated within subgroup B3. In this phylogenetic reconstruction, B-*Wolbachia* from vector insects like *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae) (EF372596) and *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) (GU563892) or other pests like *Naupactus cervinus* Boheman (Coleoptera: Curculionidae) (GQ402143) or mites like *Bryobia* spp. (i.e., EU499318) were clustered together in a well-supported clade B2. However, the *T. urticae* T2 reference sequence (EU499319) clustered within subgroup B5, which contained some populations of *T. urticae*, including those from our previous studies on which a genetic structure was devised (Aguilar-Fenollosa et al. 2012). Group B3 contained samples of *T. turkestani* and the majority of *T. evansi* Brazil population sequences. The sequence divergence of 16S rDNA among these subgroups was sometimes higher than the reference 3% used in microbiome analysis, indicating that this clustering reflects the diversity of *Wolbachia* races within Tetranychidae mites (Zhang et al. 2013).

In addition to these phylogeny-based classification methods (MLSA or 16S rDNA barcoding), other methods to identify *Wolbachia* strains have been developed in other studies (revised in Bleidorn and Gerth 2018). One of them is the MLST system, based on allele assignment of *gatB, coxA, hcpA, fbpA* and *ftsZ* genes (allele assignment was a per single nucleotide difference with reference strain in a concatenated sequence of these 5 genes) (Baldo et al. 2006; Jolley and Maiden 2010). As we only sequenced gene *ftsZ*, we could not use the whole MLST approach; however, based on this kind of study, all *T. urticae* specimens (ours and some already characterised as different) were assigned to the *ftsZ* locus 23. Whereas the Brazilian population of *T. evansi* presented the *ftsZ* locus 179. Recently, the same authors included the allele typing with only *wsp* gene due to its key features (single-copy gene, present in all Rickettsiales order, with evidence of strong stabilising selection and generally used as phylogenetic marker) and matching one of our sequenced genes (Baldo et al. 2006; Jolley and Maiden 2010). Following this *wsp* sequence typing, we were able to assign our B-*Wolbachia* sequences to different *wsp* alleles, including the description of five new *wsp* loci (submitted to the MLST database

on 9th March 2020, here presented three as X1 to X3). The *wsp* locus 61 (HVR1:18; HVR2:16; HVR3:23; HVR4:16) was the predominant one in almost all *T. urticae* feeding in citrus (54%; 66 out of 122), followed by *wsp* locus X1 (24%, HVR1:18; HVR2:16; HVR3:23; HVR4:274) in samples from *Festuca arundinacea* cover and other populations (24%). *Tetranychus urticae* feeding in *F. arundinaceae* cover showed also three other *wsp* loci (locus 370 (2.45%) = HVR1:18; HVR2:162; HVR3:23; HVR4:274; locus X2 (2.45%) = HVR1:18; HVR2:162; HVR3:23; HVR4:16; locus X3 (0.8%) = HVR1:18; HVR2:162; HVR3:23; HVR4:157) being with Mallorca population, the groups with highest diversity (each individual showed a different *wsp* allele). Due to the reduced number of individuals per population tested, we were not able to conduct a proper analysis of diversity. However, we were able to clearly identify different alleles, indicating that there exists more than one strain of *Wolbachia* in some of our populations.

Considering phylogenies and *wsp* MLST, we can conclude that *T. urticae* populations show different B-*Wolbachia* strains. Their involvement in mite's reproduction could explain the *T. urticae* population structure previously observed in Spanish citrus orchards, deserving further research to determine the link between each strain and reproductive isolation (Aguilar-Fenollosa et al. 2012, 2016; Zhang et al. 2013; Pascual-Ruiz et al. 2014). This result is in line with other studies in which *D. citri*, one of the vectors of Huanglongbing (HLB), seems to be infected by two different B-*Wolbachia* races, affecting their population structure and differential transmission of *Candidatus* Liberobacter, the plant pathogenic bacteria causing HLB (Chu et al. 2019). Similarly, *T. urticae* populations from Korean greenhouses have been reported to harbour two different *Wolbachia* races based on their *wsp* sequences, showing diverse patterns of cytoplasmic incompatibility (CI) that matched the host plant as the main phenotypic effect, similar to the population structuration previously devised due to CI in Chinese and Japanese *T. urticae* populations or in recent invasive events in Europe (Gotoh et al. 2007b; Boubou et al. 2011, 2012; Xie et al. 2011; Zhang et al. 2013; Suh et al. 2015). However, with the samples analysed, we could not relate each identified B-*Wolbachia* strain (or races) with a specific genome structuration, derived either by pest management, host plant specificity or even by its reproductive alteration pattern, which deserves further study.

Final remarks

We have identified only one bacterial species, *Wolbachia*, of the four manipulative tenant bacteria tested in our mite target populations. This bacterial species was assigned by

phylogenetic analysis to the B-supergroup, highlighting the existence of several races or strains within them. Sequence typing of *wsp* gene allowed the assignment to several alleles (with main alleles 61 and 370) and the description of five new alleles. The presence of several strains could be explained due to the biology of *Wolbachia*, either by an effect in the host reproductive strategy (population isolation) or by recent invasive events. Both hypotheses must be studied further.

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Online resources

S.I. file 1 (pdf): Supplementary information containing detailed material and methods, GenBank codes for each sequence (Table S1) and other phylogenetic reconstructions (Fig. S1–S3).

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Tables

Table 1. Mite species and other arthropods used in this work. The other arthropods were used as positive controls for *Cardinium*, *Rickettsia*, *Spiroplasma* or *Wolbachia* identification by PCR.

Order	Species	Collection locality[§]	Collection host plant (or source; known bacteria; collector)	Individuals tested
Diptera	*Drosophila melanogaster* Meigen	Valencia	(UV lab rearing, OrR strain; *Spiroplasma;* C. Garcia)	8
	D. neotestacea Grimaldi, James and Jaenike	Canada	(lab rearing; *Spiroplasma*; S. Perlman)	8
Hemiptera	*Bemisia tabaci* (Gennadius)	Israel	(lab rearing Q2 and B biotypes; *Rickettsia*; D. Santos-Garcia and S. Morin)	7
		Perelló	*Phaseolus vulgaris* L. (*Cardinium*; FJ. Beitia)	8
	Myzus persicae (Sulzer)	Tunisia	(DNA sample; *Cardinium*; R. Gil)	
Prostigmata	*Aplonobia histricina* (Berlese)	Montcada	*Oxalis pes-caprae* L.	10
	Eutetranychus banksi (McGregor)	Huelva	*Citrus lemon* L.	9
	Eutetranychus orientalis (Klein)	Málaga	*Citrus sp.*	15
	Panonychus citri (McGregor)	Betxí	*Citrus sinensis* L.	7
		Canet	*Citrus sinensis* L.	10
		Castelló	*Citrus sinensis* L.	10
		Godelleta	*Citrus sinensis* L.	25
		Mallorca	*Citrus sinensis* L.	10
		Moncofa	*Citrus sinensis* L.	10
		Montcada	*Citrus sinensis* L.	23
		Picassent	*Citrus lemon* L.	10
		Xeraco	*Citrus sinensis* L.	10
	Poliphagotarsonemus latus (Banks)	Belgium	*Rhododendron simsii* L.	80

Tetranychus evansi Baker and Pritchard	Argentina	*Solanum nigrum* L.	10
	Brazil	*Solanum nigrum* L.	15
	Murcia	*Solanum nigrum* L.	11
Tetranychus turkestani Ugarov and Nikolski	Almenara	*Cannabis sativa* L.	12
	Castelló	*Convolvulus arvensis* L.	14
Tetranychus urticae Koch	Algímia	*Citrus clementina* L.	15
	Almenara	*Citrus clementina* L.	10
	Les Alqueries	*Citrus clementina* L.	11
	Benicàssim	*Citrus clementina* L.	12
	Benifairó	*Citrus clementina* L.	3
	Betxí	*Citrus clementina* L.	3
	Castelló	*Citrus clementina* L.	52
	Castelló	*Citrus lemon* L.	3
	Castelló	*Festuca arundinacea* L.	38
	Gandia	*Citrus clementina* L.	10
	Godella	*Citrus clementina* L.	41
	Llíria	*Citrus clementina* L.	19
	Mallorca	*Citrus clementina* L.	19
	Moncofa	*Citrus clementina* L.	8
	Montcada	*Citrus sinensis* L.	24
	Montcada	*Festuca arundinacea* L.	14
	Onda	*Citrus clementina* L.	8
	Quartell	*Citrus clementina* L.	7
	Vila-real	*Citrus clementina* L.	19
	Vinaròs	*Citrus clementina* L.	52

[§]Spanish localities, unless another country is indicated.

Table 2. Universal and bacterial diagnostic primer pairs sequence, amplicon size, annealing temperature and references, used to determine the incidence of bacterial symbionts in our samples.

Target	Primer name	Sequence (5'→3')	Amplicon size (pb)	T_A (°C)	V_R (µl)	C_{Mg2+} (mM)	Primers references
18SrDNA	18Sup_1060	AGT TAG AGG TTC GAA GGC GAT CAG	233	55	25	2.5	Monzó et al. 2010
	18Slo_1270	TGG TAA GTT TTC CCG TGT TGA GTC					
16SrDNA	Univ_16S_8F	AGA GTT TGA TCM TGG CTC AGA TTG	1200	60	25	1.5	van Ham et al. 1997
	Univ_16S_1507R	TAC CTT GTT AYG ACT TCA CCC CAG					
Cardinium (16S rDNA)	CLO_F1_16S	GGA ACC TTA CCT GGG CTA GAA TGT ATT	450	57	20	1.5	Zhao et al. 2013
	CLO_R1_16S	GCC ACT GTC TTC AAG CTC TAC CAA C					
Rickettsia (16S rDNA)	Rb_F	GCT CAG AAC GAA CGC TAT C	880	58	25	2.5	Gottlieb et al. 2006; Kliot et al. 2014
	Rb_R	GAA GGA AAG CAT CTC TGC					
Spiroplasma (16S rDNA)	Spoul-F	GCT TAA CTC CAG TTC GCC	450	55	25	2.5	Montenegro et al. 2000; Osaka et al. 2013
	Spoul-R	CCT GTC AAT GTT AAC CTC					
Wolbachia (16S rDNA)	99F	TTG TAG CCT GCT ATG GTA TAA CT	900	52	25	1.5	O'Neill et al. 1992
	994R	GAA TAG GTA TGA TTT TCA TGT					
ftsZ (*Wolbachia*)	Wo_FtsZuniF	GGY AAR GGT GCR GCA GAA GA	770	54	20	1.5	Lo et al. 2002
	Wo_FtsZuniR	ATC RAT RCC AGT TGC AAG					
wsp (*Wolbachia*)	81F	TGG TCC AAT AAG TGA TGA AGA AAC	610	55	25	1.2	Braig et al. 1998
	691R	AAA AAT TAA ACG CTA CTC CA					

FIGURE LEGENDS

Fig. 1 Bar chart of *Wolbachia* incidence, as the percentage of individuals who tested positive from the total, per species. Note that only one population of *Panonychus citri* showed *Wolbachia* infection (0.87%). The total number of individuals tested are, in order of species appearance and from left to right, 10, 9, 15, 115, 80, 36, 26 and 368.

Fig. 2 Phylogenetic inference from MLSA (containing 16S rDNA, *ftsZ* and *wsp*, concatenated sequences) of 72 *Wolbachia* specimens (indicated with the corresponding species name, sample code and GenBank accession number of the 16S sequence of each specimen) inferred using the following: (A) Bayesian analysis or (B) Maximum Likelihood (ML) method. Evolutionary distances were computed using the Tamura 3-parameter method after modelling rate variation among sites with a gamma distribution (shape parameter = 0.08). All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA X. *Wolbachia* supergroups are indicated in the krone section outside with different patterns.

Fig. 3 Phylogenetic inference from MLSA (containing only 16S rDNA and *ftsZ* concatenated sequences) of 90 *Wolbachia* specimens (indicated with the corresponding species name, sample code and GenBank accession number of the 16S sequence of each specimen) was inferred using the following: (A) Bayesian analysis or (B) Maximum Likelihood (ML) method. Evolutionary distances were computed using the Tamura 3-parameter method after modelling rate variation among sites with a gamma distribution (shape parameter = 0.27). Evolutionary analyses were conducted in MEGA X. *Wolbachia* supergroups are indicated in the krone section outside with different patterns.

Fig. 4 Phylogenetic inference, using only the *wsp* gene of 145 *Wolbachia* specimens (indicated with the corresponding species name, sample code and GenBank accession number), was performed using the Bayesian analysis under the GTR + I + Γ model of DNA substitution. *Wolbachia* supergroups are indicated in the krone section outside with different patterns.

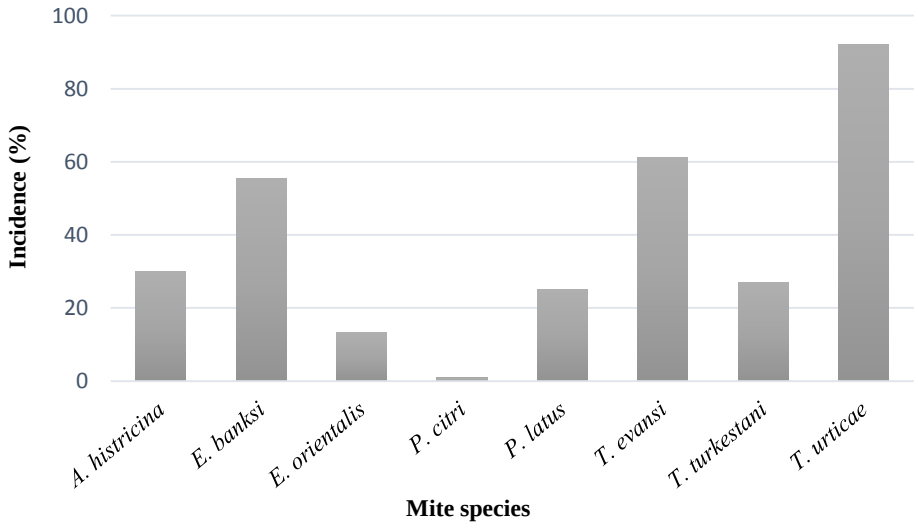
Fig. 5 Phylogenetic inference, using only the *FtsZ* gene of 112 *Wolbachia* specimens (indicated with the corresponding species name, sample code and GenBank accession

number), was performed using the Bayesian analysis under the GTR + I + Γ model of DNA substitution. *Wolbachia* supergroups are indicated in the krone section outside with different patterns.

Fig. 6 Phylogenetic inference, using only the 16S region of 126 *Wolbachia* specimens (indicated with the corresponding species name, sample code and GenBank accession number), was performed using the Bayesian analysis under the GTR + I + Γ model of DNA substitution. *Wolbachia* supergroups are indicated in the krone section outside with different patterns.

745

746 **Fig. 1**



747

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749

Fig. 2

(A)

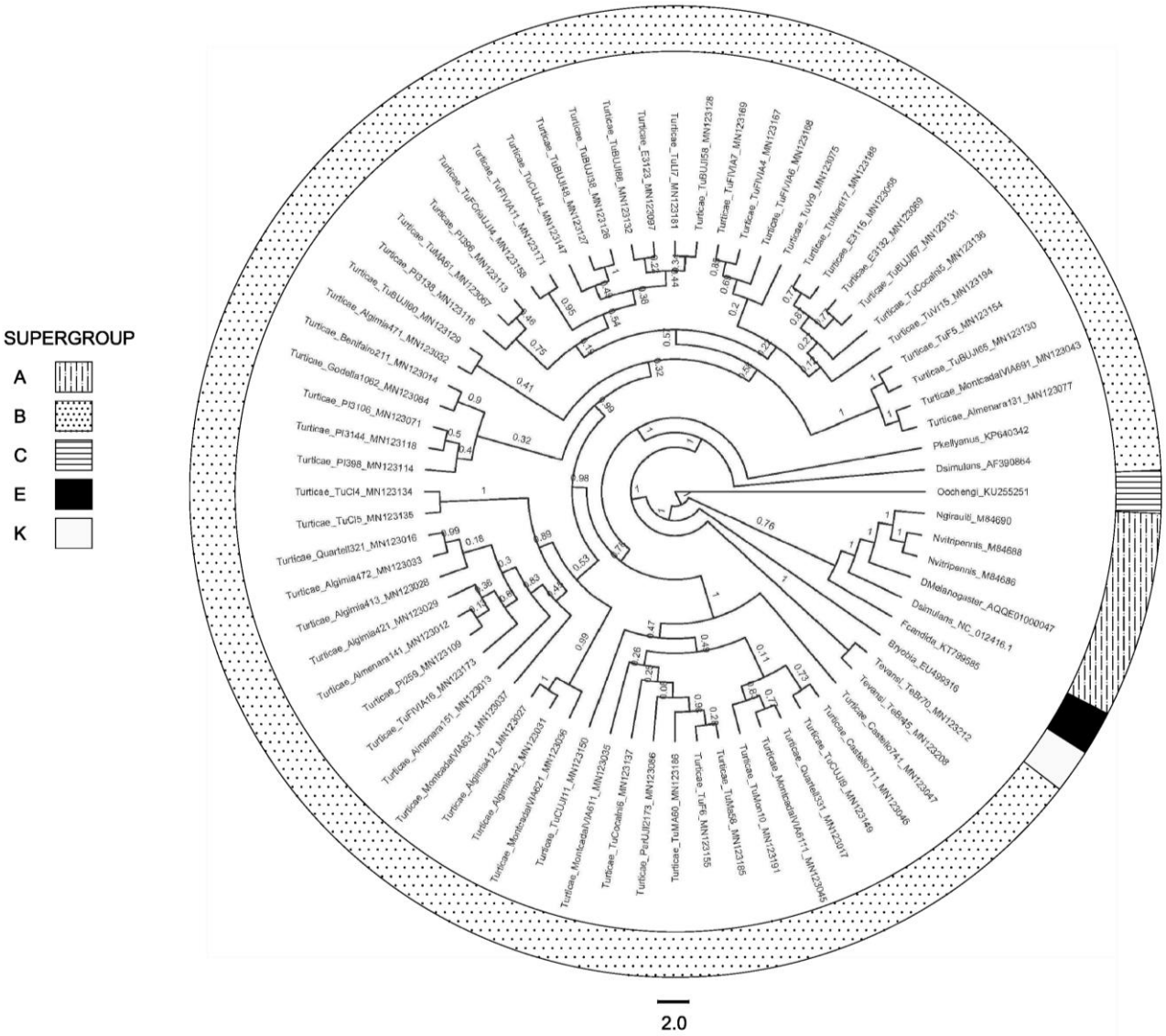

Fig. 2

(B)

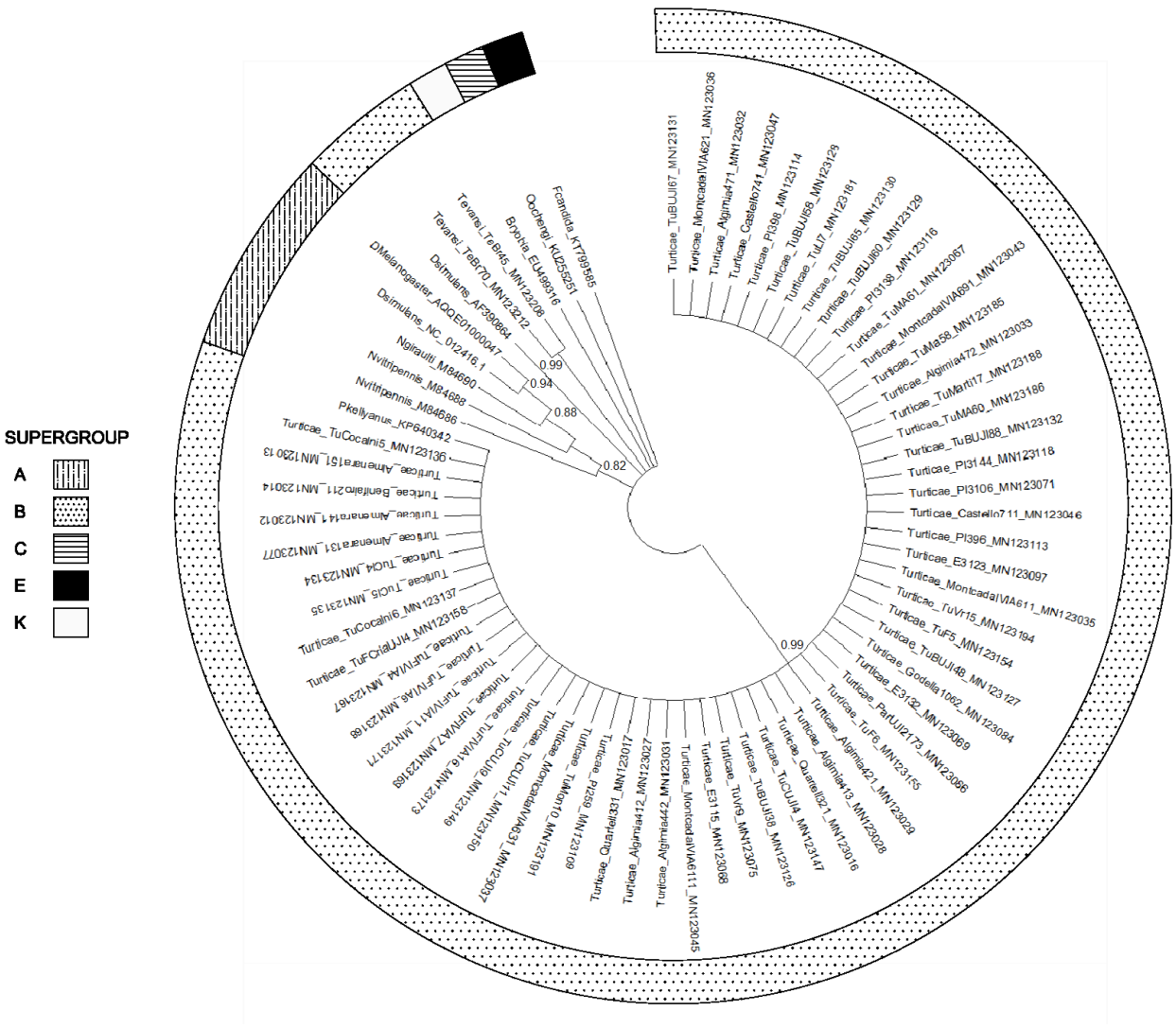
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Fig. 3

(A)

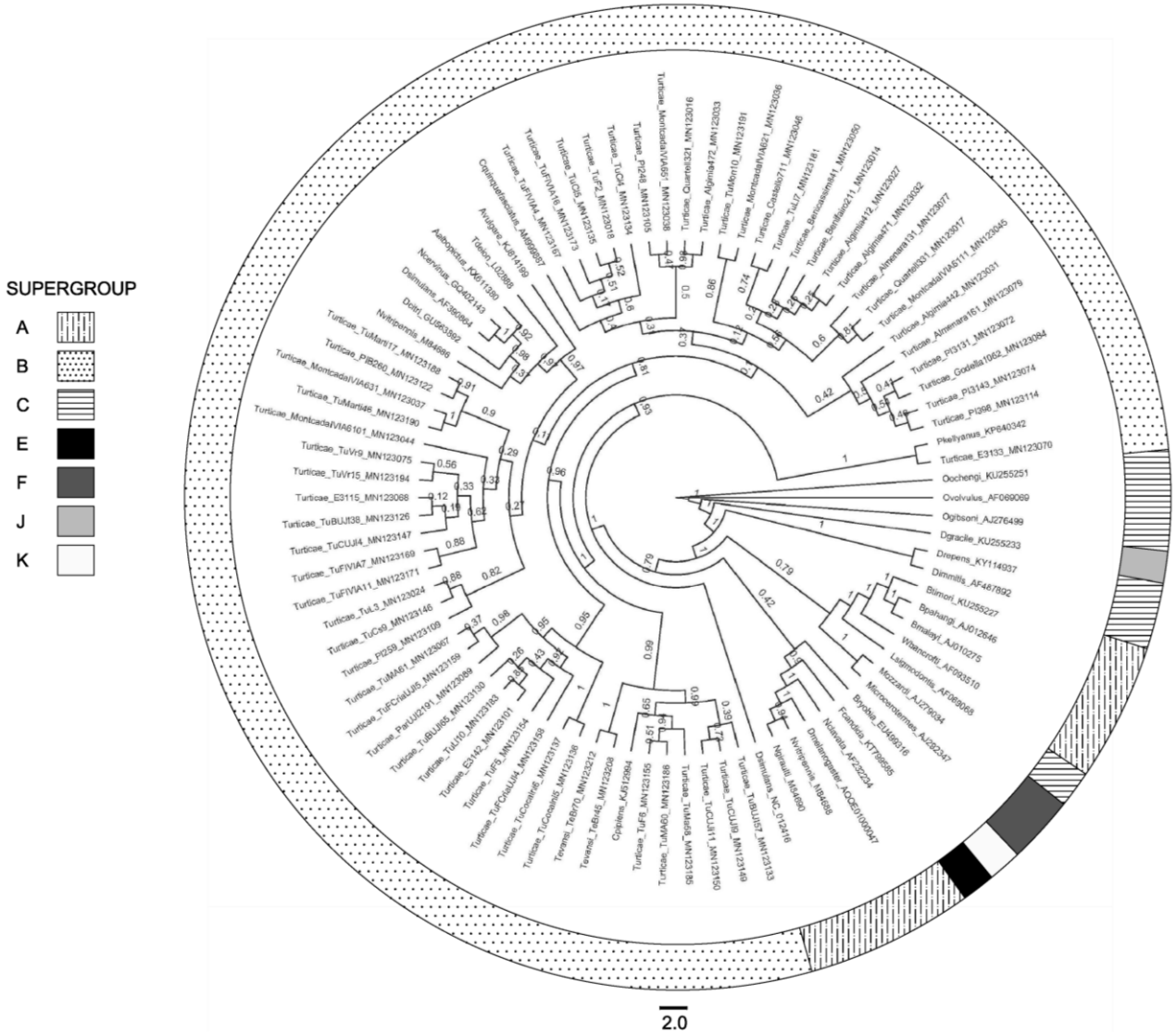

Fig. 3

(B)

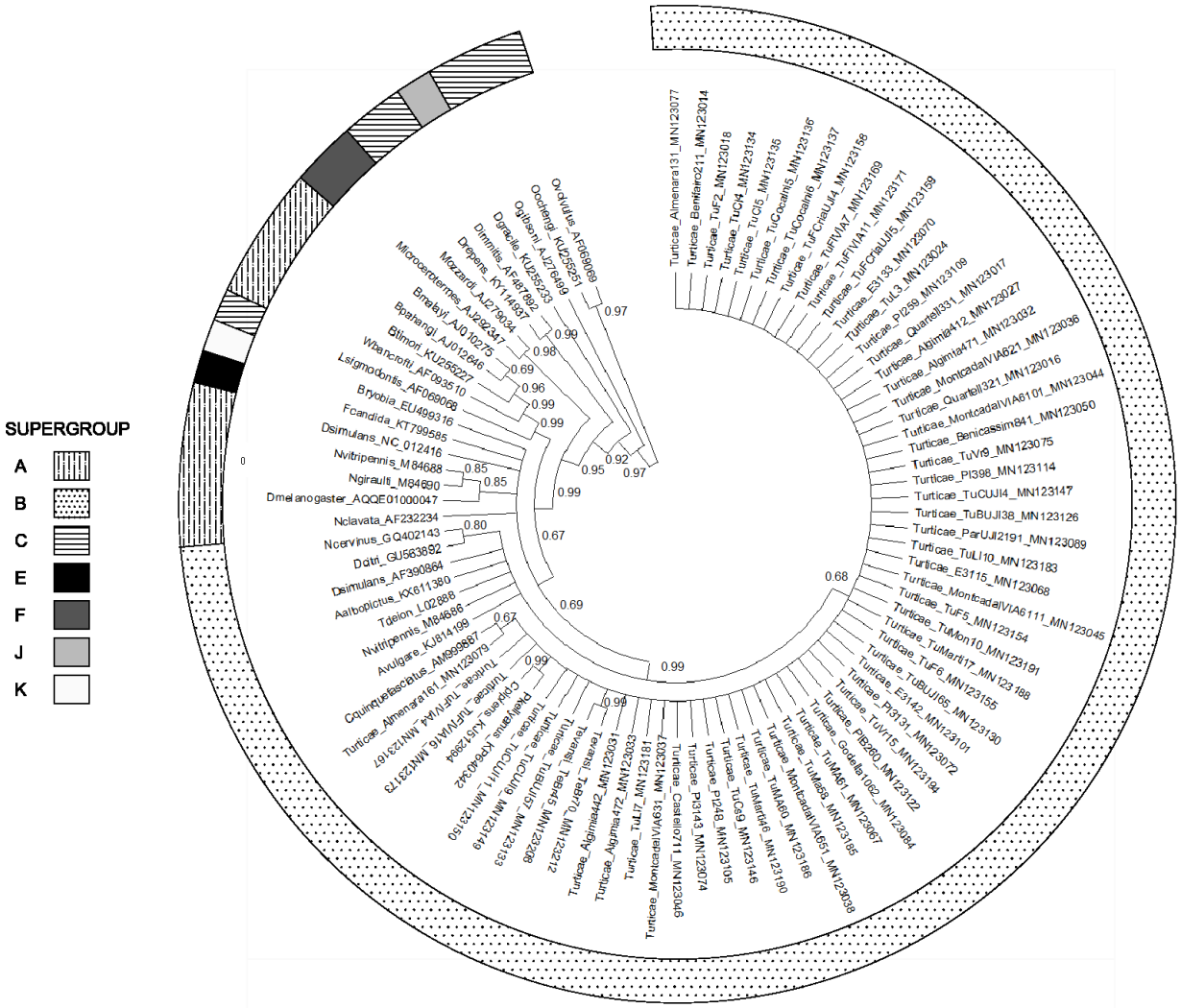
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Fig. 4

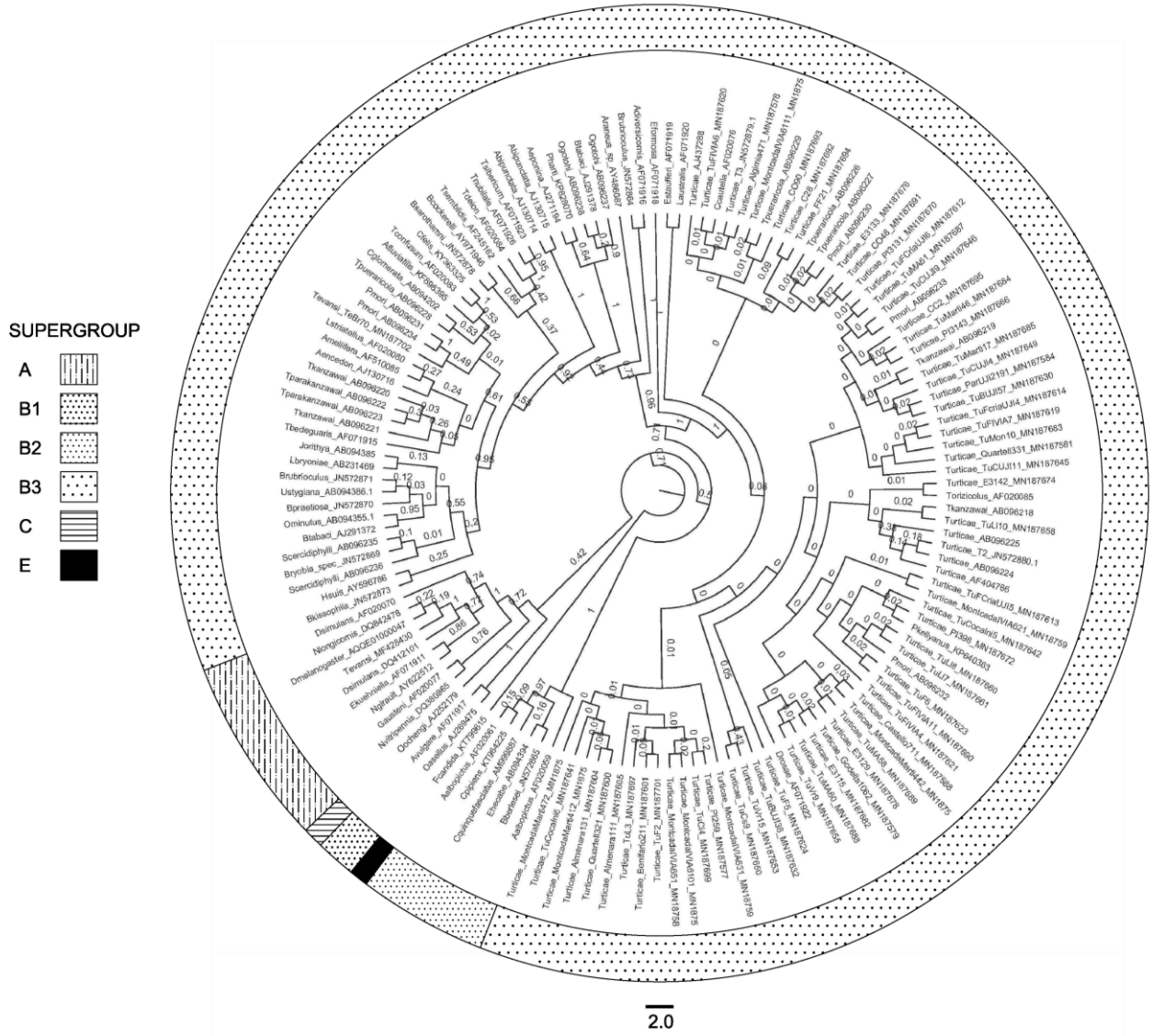

Fig. 5

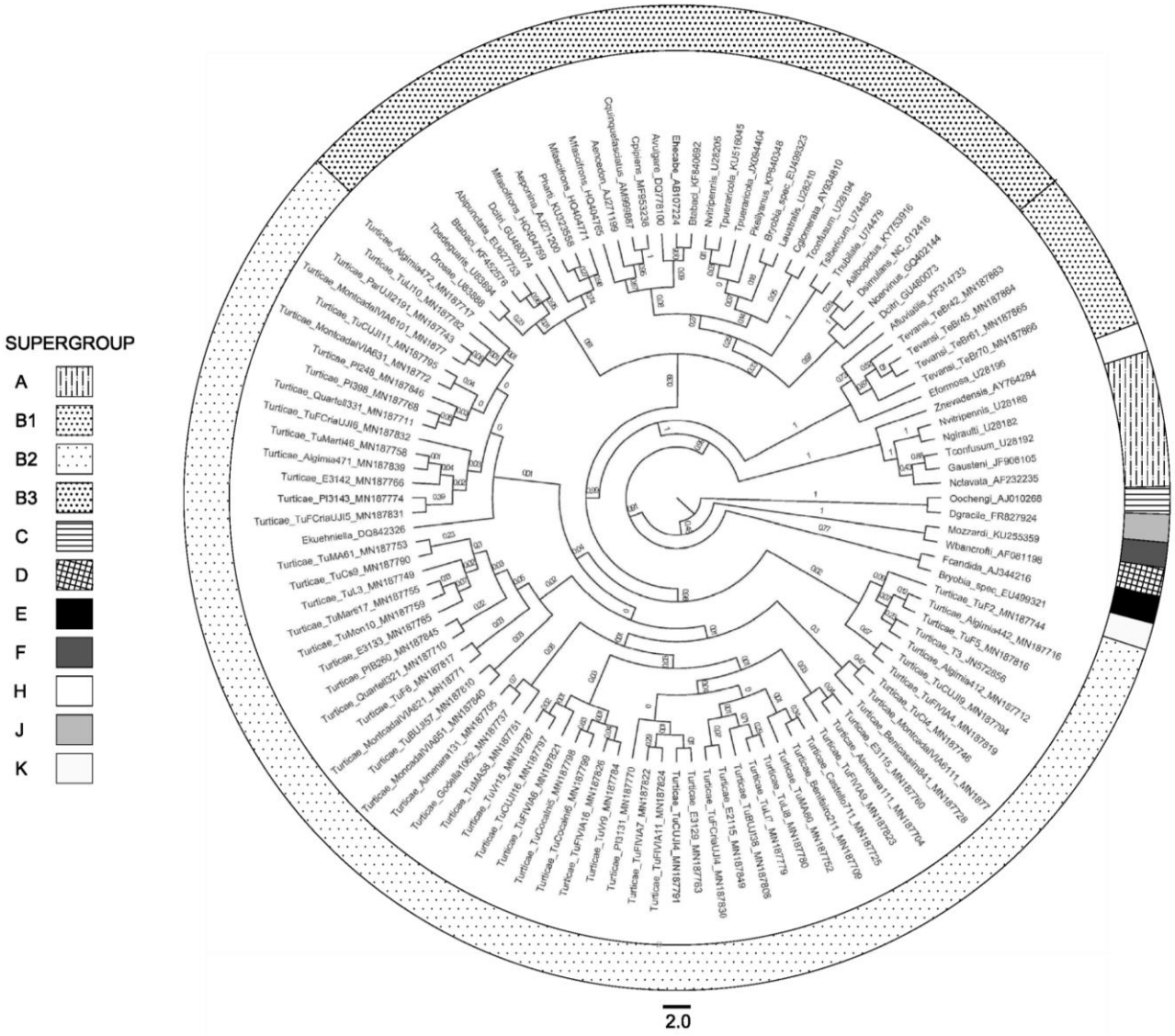

Fig. 6