Portiera gives new clues on the evolutionary history of whiteflies

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Abstract

Whiteflies (Hemiptera: Sternorrhyncha: Aleyrodidae) are a superfamily of small phloem-feeding insects. Their taxonomy is currently based on the morphology of nymphal stages that display phenotypic plasticity, which produces inconsistencies. To overcome this limitation, we developed a new phylogenetic framework that targets five genes of Candidatus Portiera aleyrodidarum, the primary endosymbiont of whiteflies. Portiera lineages have been co-diverging with whiteflies since their origin and therefore reflect their host evolutionary history. We also studied the origin of stability and instability in Portiera genomes by testing for the presence of two alternative gene rearrangements and the loss of a functional polymerase proofreading subunit (dnaQ), previously associated with genome instability. We present two phylogenetic reconstructions. One using the sequences of all five target genes from 22 whitefly species belonging to 17 genera. The second uses only two genes to include additional published Portiera sequences of 21 whitefly species, increasing our sampling size to 42 species from 25 genera. The developed framework showed low signal saturation, specificity to whitefly samples, and efficiency in solving inter-genera relationships and standing inconsistencies in the current taxonomy of the superfamily. Genome instability was found to be present only in the Aleurolobini tribe containing the Singhiella, Aleurolobus and Bemisia genera. This suggests that Portiera genome instability likely arose in the Aleurolobini tribe’s common ancestor, around 70 Mya. We propose a link between the switch from multi-bacteriocyte to a single-bacteriocyte mode of inheritance in the Aleurolobini tribe and the appearance of genome instability in Portiera.

Keywords: whitefly systematics, long-enduring taxon, divergence dating, genome stasis, molecular evolution, whitefly development.

1 Introduction

The order Hemiptera is the largest monophyletic group of hemimetabolous (without metamorphosis) insects and is, characterized by specialized piercing-sucking mouth-parts (“rostrum”) (Grimaldi and Engel, 2005). The hemipteran order is divided into four suborders: Sternorrhyncha, Auchenorrhyncha, Heteroptera, and Coleorrhyncha. Most hemipteran insects feed on plant sap (phloem and xylem), but some heteropteran insects are predatory (Grimaldi and Engel, 2005; Cryan and Urban, 2012). The Sternorrhyncha suborder is usually divided into the Aphidinea lineage containing the Aphidoidea (aphids) and Coccoidea (scale insects) superfamilies, and the Psyllidea lineage containing the Psyl-
loidea (psyllids) and Aleyrodoidea (whiteflies) superfamilies (Shcherbakov, 2000).

The Aleyrodoidea superfamily is composed of one family, the Aleyrodidae, which is divided into two extant subfamilies, the Aleurodicinae and the Aleyrodinae, and an extinct one, the Berneainae. The Aleurodicinae subfamily is composed of 20 genera, mainly distributed in Neotropical/Australasian regions (Charles, 2010). The Aleyrodinae, with 140 described genera, is a more diverse and globally distributed (Manzari and Quicke, 2006). It includes the major pest species *Bemisia tabaci* and *Trialeurodes vaporariorum*. Although the extant whitefly subfamilies were reported to have their origin in the Middle Cretaceous (Campbell et al., 1994), the first fossils of the present extant families were dated to the Lower Cretaceous (Drohojowska and Szwedo, 2015). During that period, whiteflies were associated with gymnosperm forests and/or pro-angiosperms, in contrast to extant whitefly species, which feed mainly on angiosperms. It is assumed that the emergence of angiosperms in the Lower Cretaceous has promoted the radiation of the whitefly superfamily due to the opening of new environmental niches. This event triggered their diversification and speciation along with the angiosperms (Middle-Upper Cretaceous), leading to the emergence of the modern whitefly species (Drohojowska and Szwedo, 2015).

Whiteflies are the less diverged superfamily among the Sternorrhyncha. Their taxonomy is based mainly on the fourth instar nymph morphology, which is quiescent and known as “puparium”, instead of the more commonly used adult phase (Martin and Mound, 2007; Evans, 2007). The “puparium” stage presents phenotypic plasticity with phenotypes affected both by the identity of the host plants and different abiotic factors (Manzari and Quicke, 2006; Martin and Mound 2007). Also, most of the phylogenetic research so far has focused on relatively few species with significant economic impacts, such as the *B. tabaci* species complex (Brown et al., 1995; Frohlich et al., 1999; Boykin et al., 2007; De Barro et al., 2011; Lee et al., 2013; Hsieh et al., 2014; Wang et al., 2014). To the best of our knowledge, only four published studies have tried to establish broad phylogenetic relationships among the different whitefly genera. Three of them were based on molecular phylogenetics (Thao and Baumann, 2004a; Ovalle et al., 2014; Dickey et al., 2015) while the third utilized morphological cladistics (Manzari and Quicke, 2006). Thao and Baumann (2004a) used Portiera’s 16S and 23S rRNA genes to classify 20 species from 12 genera and reported some incongruence between taxonomical names and molecular data. Both Ovalle et al. (2014) and Dickey et al. (2015) works used the 5’ region of the mitochondrial cytochrome oxidase 1 (*mtCOI*) barcode to classify 20 species from 9 genera and 104 species from 24 genera, respectively. However, both studies showed low support at inner nodes and highlighted the limited capacity of the *mtCOI* barcode to establish inter-genera relationships. More recently, Manzari and Quicke (2006) used 94 morphological characters present in 439 whitefly species (from seven Aleurodicinae and 117 Aleyrodinae genera) to reconstruct the species phylogenetic relationships. The authors conclude that in many cases, the ability of the “puparium” morphological characters to provide species definition is likely to be limited and might not even be genera specific. Therefore, it is clear that the classification and diversity of whitefly superfamily need a reassessment.

Most sternorrhynchan insects, including whiteflies, present obligatory bacterial symbionts harbored within specialized cells, termed bacteriocyte. These primary intracellular symbionts, commonly named P-endosymbionts, generally complement their hosts’ restricted diets (plant sap) (Hansen and Moran, 2014). P-endosymbionts exhibit mother-to-offspring vertical transmission, which promotes their co-speciation with their hosts and enable them to reflect their hosts’ phylogenetic history (co-cladogenesis) (De Vienne et al., 2013). The genome of P-endosymbionts has been reduced to a
basic set of genes responsible for maintaining the symbiotic relationship and minimal cell machinery. Other characteristics include the absence (nearly) of gene duplications, mobile elements, and acquisition of new genetic material (no gene flow) (Latorre and Manzano-Marín 2016). These characteristics make the genomes of P-endosymbionts a valuable resource for reconstructing their hosts phylogenetic relationships, as already have been demonstrated in aphids (Martinez-Torres et al. 2001; Jousselin et al. 2009; Nováková et al. 2013; Meseguer et al. 2015, 2017) and psyllids (Hall et al. 2016).

The P-endosymbiont of whiteflies is Candidatus Portiera aleyrodidarum (hereafter Portiera) (Thao and Baumann, 2004b). Portiera forms a monophyletic clade together with Ca. Carsonella ruddii, the P-endosymbiont of psyllids. Based on molecular data, it has been proposed that the ancestral symbiosis was established in the Psyllinea lineage, before their divergence into the Aleyrodoidea and Psylloidea lineages (Santos-Garcia et al. 2014). Therefore, Portiera has been co-diverging with their whitefly hosts since their origin, reflecting this way both the hosts’ phylogenetic relationships and divergence time (Santos-Garcia et al. 2015). Until now, only three Portiera genomes from species others than B. tabaci have been sequenced. As other P-endosymbionts, these three Portiera have maintained a genome stasis for more than 135 million years (Myr) (Sloan and Moran 2013; Santos-Garcia et al. 2015). In contrast, Portiera genomes from the B. tabaci species complex, although syntenic among themselves, have lost the synteny with the other three published Portiera genomes. The genome rearrangements of Portiera from B. tabaci is correlated with a massive loss of genes required for correct DNA replication and the repair machinery. These losses include the DNA polymerase III subunit epsilon dnaQ, which is required for repairing spontaneous mutations (proofreading activity) (Sloan and Moran 2013; Santos-Garcia et al. 2015). Currently, it is not clear if the above mentioned genome instability is a unique characteristic of Portiera from B. tabaci or a more general phenomenon present in other lineages.

In this work, we explored the use of Portiera as a tool for determining relationships among whiteflies. First, we used up to five Portiera genes to reconstruct the phylogeny and divergence of 42 whitefly species belonging to 25 different genera. Freshly collected, ethanol stored, and museum exsiccate samples were used to ensure our framework’s robustness. Our analysis indicated that phylogenetics based on Portiera gene sequences is a promising approach as it shows low signal saturation and can efficiently solve inter-genera relationships. Second, we used genome sequencing and PCR amplification to screen for genome rearrangements and the presence/absence of a functional dnaQ gene in Portiera to understand better the origin of the genome instability found in some Portiera lineages. Our analysis indicated that Portiera genome instability originated once in the Aleurolibini tribe ancestor. The possible link between a newly emerged mode of bacteriocyte transmission in the Aleurolibini tribe and the appearance of genome instability is discussed.

2 Material and Methods

Whiteflies collections and gDNA extraction

A total of 29 samples, accounting for 25 different whitefly species, were obtained. Whitefly samples were from different sources: freshly collected and stored in ethanol until use (adult insects), Prof. Dan Gerling’s collection (adult insects conserved in ethanol), and exsiccate collection samples from the Natural History Museum (NHM) in London (nymphs were removed from dry leaves and sent in ethanol). Before any genomic DNA extraction was performed, five adult insects (or nymphs from the NHM collection) were rehydrated by
consecutive passes in 70%, 50%, 30%, 0% v/v ethanol solutions in sterile water. Whiteflies were transferred to a new 1.5 ml tube containing 80µl lysis buffer T1 and were homogenized with 1.4 mm zirconia beads (CK14, Bertin Instruments) using a bead-beater (Minilys, Bertin Instruments). Genomic DNA (gDNA) was extracted with NucleoSpin Tissue XS (Macherey-Nagel) following the manufacturer instructions. For the NHM samples, a nondestructive method was used whenever possible. Nymphs were incubated overnight (56°C) in 80µl lysis buffer T1 and 8µl Proteinase K (20µg/µl). gDNA was extracted from the lysis buffer using the NucleoSpin Tissue XS standard protocol. The nymphs were recovered, cleaned with sterile water, and stored in fresh ethanol. gDNA from seven samples that had less than five individuals were subjected to whole-genome amplification (GenomiPhi V2, GE Healthcare), following manufacturer instructions, to ensure sufficient material. For Illumina sequencing, Singhiella simplex adults were accidentally collected together with Pealius mori adult whiteflies in July 2018 from Ficus benjamina (GPS coordinates 31.904511;34.804562) and stored in ethanol. Later on, whiteflies were rehydrated and sexed. Female abdomens (50) were dissected under a stereo-microscope using autoclaved 1X Phosphate-buffered saline. Abdomens were homogenized with a bead-beater, and gDNA was extracted with NucleoSpin Tissue XS, as described above. Whole-genome shotgun sequencing was performed in a NovSeq 6000u sing a TruSeq DNA PCR Free Library (2x150bp) at Macrogen Europe.

PCR screening, sequencing, and ancestral estate reconstruction

For the PCR screening, five genes present in all insect endosymbionts showing extremely reduced genomes were selected (Moran and Bennett, 2014). These genes are widely used as bacterial phylogenetic markers and includes the 16S and 23S ribosomal RNAs (rRNAs), the chaperons groEL and dnaK, and the RNA polymerase sigma factor rpoD. We manually designed Portiera specific universal primers using available Portiera genomes from both the Aleyrodinae (B. tabaci and T. vaporariorum) and Aleurodinae (Aleuridius dispersus and A. floccissimus) subfamilies in UGENE v1.28.1 (Okonechnikov et al., 2012) (Table S1). Primers melting temperature (Tm), off-targets, and possible primer-dimer interactions were computed with Primer3 software implemented on https://eu.idtdna.com/calc/analyzer. Primers (0.5 mM each) were mixed with the KAPA2G Robust HotStart ReadyMix (Kapa Biosystems) inside a DNA/RNA UV-Cleaner cabinet (UVC/T-AR). PCR was performed using the following general profile: 95°C for 5 min, [95°C for 30 sec, Tm°C for 15 sec, 72°C for 1 min]x35, 72°C for 5 min. Annealing temperature (Tm) was set up for each primer set according to Primer3 predictions (Table S1). When required, the temperature was adjusted trying 5°C above or below of the predicted Tm.

PCR product size was confirmed by electrophoresis using 1% agarose gel, purified with DNA Clean & Concentrator 5 (Zymo Research), and sequenced by Sanger technology in both directions at Macrogen Europe. For each amplicon, sequences were quality screening/clipping and their consensus alignment performed with the Staden Package (Bonfield and Whitwham, 2010). In parallel, we designed primers that target the DNA polymerase III subunit epsilon dnaQ. Also, we targeted two regions with different gene order in Portiera of B. tabaci, lepA-groEL (A_Bt) and secA-leuC (B_Bt), compared to the ancestral gene order found in other sequenced Portiera, groEL-tpsA (A) and leuC-leuD (B). Primer design and PCR reactions were conducted as described above using the predicted Tm (Table S1). PCR products were visualized by electrophoresis using a 1%. Some obtained amplicons were Sanger sequenced to validate the amplified region. Additionally, for each whitefly species collected, the 5’ region of the mitochondrial cytochrome oxidase 1 (mtCOI) gene was am-
plified using the universal primers LCO1490 (F) and HCO2198 (R) (Folmer et al., 1994). In cases where this set of primers failed to amplify, the C1-J-2195 (F) and L2-N-3014 (R) primer set targeting the 3’ region was used (Frohlich et al., 1999). In species where both sets of primers failed to amplify and mtCOI sequences were available at public databases, species-specific sets were designed (Table S1). PCR conditions and procedures were the same as described above.

**Portiera lineages Divergence Dating**

Two datasets were used to infer the divergence time of the whitefly species analyzed. The first dataset incorporated sequences of Portiera 16S and 23S rRNA genes amplified in this study, 16S and 23S rRNA gene sequences generated by Thao and Baumann (2004b), and 16S and 23S rRNA gene sequences extracted from the downloaded transcriptomes/genomes. The final dataset contained 59 sequences from 45 different species (including six belonging to the B. tabaci species complex). The second dataset integrated the sequences of the 16S and 23S rRNA genes with those of the three coding genes: dnaK, rpoD, and groEL. It contained 32 sequences from 29 whitefly species, mostly obtained in this study plus few that were acquired from public transcriptomes/genomes. In both datasets analysis, orthologous genes extracted from Chromohalobacter salexigens DSM3043 (NC_007963.1) were used as outgroups. The 16S and 23S rRNA genes were aligned with R-Coffee v11.00.8cbe486 (-mode=rmcoffee -iterate=100) (Notredame et al., 2000) and pruned with Gblocks v0.91b (-t=d -b5=h) (Castresana, 2000). The three coding genes (dnaK, rpoD, and groEL) were codon-aligned with MACSE v2.03 (-prog alignSequences -gc_def 11) (Ranwez et al., 2018) and pruned with Gblocks v0.91b (-t=c -b5=h). The 19 obtained mtCOI gene sequences (5’ region) were aligned in the same way but using the invertebrate mitochondrial code in MACSE v2.03 and no gaps allowed in Gblocks v0.91b. Substitution saturation was assessed using the pruned alignments as an input for Xia’s test implemented in DAMBE v7.2.3 (Xia, 2018) (executed under wine v1.6.2-0ubuntu14.2). BEAST v2.5.2 (Bouckaert et al., 2014) was used to infer a Bayesian posterior consensus tree and the divergence time of the different nodes as previously described (Santos-Garcia et al., 2015). Detailed procedures of divergence dating can be found at Supplementary Material and Methods.

**Whole Genome Shotgun sequencing, genome assembly and annotation of the Singhiella simplex and Pealius mori joint sample**

NovaSeq sequencing produced 75,274,888 raw reads that were quality screened with Trimmomatic v0.33 (same parameters as described above). Possible polyGs produced by the NovaSeq platform were trimmed with fastp v0.19.7 (Chen et al., 2018). Cleaned reads were classified with Kraken v2.0.6-beta and the custom database described above. All reads assigned to Portiera, Halomonadaceae, or Oceanospirillales were extracted and assembled with SPAdes v3.13.0 (-sc -careful) (Bankevich et al., 2012). Three contigs larger than 60Kb (385Kb in total) and ~100x coverage plus several contigs between 80Kb and 5Kb (420Kb in total) and ~600X coverage were recovered. Kraken2 classification and coverage suggested two putative Portiera populations. To screen for possible Portiera other than that of Singhiella simplex, all sequences obtained during the PCR screening were used as a query in a BLASTN search against the obtained contigs. BLASTN results confirmed that two different Portiera genomes were present. Some large contigs with ~100x coverage had perfect match to the Portiera amplified genes from Pealius mori. Several smaller contigs with a coverage of ~600X had perfect match to the amplified Portiera genes.
from *S. simplex*. This confirmed that some *P. mori* individuals were collected together with *S. simplex*, probably due to the ability of both whitefly species to exploit *Ficus benjamina* as a host-tree.

As a result, the Kraken2 database was rebuilt including the obtained contigs, and cleaned reads were re-classified. *Portiera* reads were re-assembled separately according to their whitefly host with SPAdes v3.13.0. SSPACE v3 ([Boetzer et al., 2011](https://doi.org/10.1093/bioinformatics/btp373)) and GapFiller v1.10 ([Boetzer and Pirovano, 2012](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3469145/)) were used for scaffolding and gap-filling of the re-classified reads. Gap5 from the Staden package was used to evaluate the assemblies quality, to detect the presence of chimeras and miss-assemblies, to join contings manually (when possible), and to check for circular contigs. The first genome to be assembled was that of *Portiera* from *P. mori*. It produced a closed circular contig without requiring any iterative mapping step. In contrast, the *Portiera* genome from *S. simplex* remained as 9 contigs after several rounds of iterative mapping, discarding at each round every sequence (if present) with a significant (90% identity threshold) match to the *Portiera* genome of *P. mori*. In brief, iterative mapping was run as follows: cleaned reads were mapped against the assembled contigs of *S. simplex* with Bowtie v2.3.5.1 ([Langmead and Salzberg, 2012](https://doi.org/10.1093/bioinformatics/bts200)). Reads without a minimum overlap of 50% and 90% identity to the contigs were discarded. Surviving reads were added to the pool of putative *Portiera* reads from *S. simplex*. The reads were mapped to the contigs of *S. simplex* with MIRA v4.9.6 ([Chevreux, B., Wetter, T. and Suhai, 1999](https://doi.org/10.1006/bion.1998.2013)), and then imported to Gap5 for manual joining/gap closure. Both final assemblies were corrected with Pilon v1.23 (–fix all,amb) ([Walker et al., 2014](https://doi.org/10.1093/molbev/msu122)) and the clean classified reads. Finally, the annotation of the genomes was performed with prokka v1.14.5 ([Seemann, 2014](https://doi.org/10.1186/s13059-014-0049-1)), using all available *Portiera* genomes for building the protein database of primary annotation (–protein). Obtained annotations were manually inspected and curated in Artemis v1.5 ([Rutherford et al., 2000](https://doi.org/10.1080/136727999308200)).

**Portiera lineages Comparative Genomics**

Proteomes of *Portiera* from *S. simplex* (ERZ1272841), *P. mori* (ERZ1272840), *B. tabaci* species - MEAM1 (NC_018677.1), MED (NC_018676.1), and Asia II 3 (NZ_CP016327.1), *T. vaporariorum* (LN649236.1), *A. dispersus* (LN649255.1) and *A. floccissimus* (LN734649.1) were extracted with a custom python script. Orthologous clusters of proteins (OCPs) were calculated with OrthoFinder v2.3.3 (-M msa -S mmseqs -T iqtree) ([Emms and Kelly, 2019](https://doi.org/10.1093/molbev/msy023)). Obtained OCPs were manually curated based on protein annotations. Shared and specific OCPs were plotted with UpsetR ([Conway et al., 2017](https://doi.org/10.1093/molbev/msy023)). Synteny between *Portiera* genomes, based on 230 single-copy core OCPs (from 235), was plotted with genoPlotR ([Guy et al., 2010](https://doi.org/10.1093/molbev/msa111)). Finally, metabolic potential comparisons were performed with Pathway Tools v23.5 ([Karp et al., 2002](https://doi.org/10.1093/molbev/msa111)).

Curated OCPs were converted into a binary matrix (presence/absence) and species-specific OCPs annotated as hypothetical proteins were discarded (21 OCPs). The binary matrix and the species tree obtained with OrthoFinder v2.3.3 were used as inputs for COUNT v10.04 ([Csuos, 2010](https://doi.org/10.1016/j.cub.2010.01.066)) to reconstruct the gene losses history during *Portiera* evolution. The reconstruction was performed under a posterior algorithm and allowed only gene losses. Results from the dnaQ screening, codified as a binary matrix, were used together with the Bayesian trees obtained (the tree based only on two rRNA genes and the tree based on the five gene set) as an input for the Ancestral Character Estimation (ace) function implemented in ape (R package) ([R Core Team, 2018](https://doi.org/10.1093/molbev/msy023)), ([Paradis and Schliep, 2019](https://doi.org/10.1093/molbev/msy023)). The presence of dnaQ on the internal nodes from both datasets was estimated using a maximum likelihood (ML) approach as a discrete character and a model assuming only gene losses. Phylogenetic trees with dnaQ presence probabilities were plotted with ape.
**Portiera** lineages Molecular Evolution

Synonymous ($dS$) and non-synonymous ($dN$) substitution ratios, nucleotide substitutions per site per year ($dS/t$ and $dN/t$), and omega ($\omega$) values were calculated as previously described in Santos-Garcia et al. (2015) using Codeml from PAML v4.7 package (Yang, 2007). The full procedure can be found at Supplementary Material and Methods.

Repeats and intergenic regions analysis

Repeats annotation and intergenic regions comparative analysis can be found at Supplementary Material and Methods.

Transcriptome and Mitochondrion assembly, data retrieval, and mitochondrial molecular evolution

Dialeurodes citri and B. tabaci SSA1 transcriptome assembly procedures, mitochondrial genomes assembly and annotation of S. simplex and P. mori, and Portiera genomes and whiteflies’ mitogenomes retrieval can be found at Supplementary Material and Methods.

Establishing Portiera phylogenetic relationships and divergence dating

The number of taxa and genes that are used in phylogenetic analysis can affect their outcome (Nabhan and Sarkar, 2012). For that reason, we conducted two phylogenetic reconstructions. One that used the sequences of all five target genes in Portiera: 16S and 23S rRNA, dnaK, rpoD, and groEL. The second analysis used only the two rRNA genes. This allowed us to add sequences of 20 whitefly species from 13 genera that were reported by Thao and Baumann (2004a).

3 Results

**Portiera** marker genes amplification

gDNA was extracted from 26 of the 29 collected samples, standing for 22 whitefly species from 17 genera (Table S1). We failed to obtain gDNA from three NHM collection exsiccate samples (Table S1), even when applying a whole genome amplification approach. The five sets of primers that target Portiera genes successfully amplified in 25 samples. One sample, Bemisia reyesi JHM 7496, was excluded from further analysis because we could not amplify the target regions of the 23S rRNA and rpoD genes. The primers sets targeting the mtCOI gene had lower efficiency and produced the expected PCR fragments in 18 of the 26 samples. The LCO1490/HCO2198 set produced a PCR fragment in 15 samples but two of them were parasitoid wasp sequences instead of whitefly sequences. The C1-J-2195/L2-N-3014 produced a PCR fragment in one sample. The other four successful amplifications used species-specific mtCOI primers. The five amplified Portiera genes did not show signatures of substitution saturation in their phylogenetic signal, and presented saturation values that were lower than those calculated for the full set of mitochondrial genes including the mtCOI amplicon (Table S3). In fact, the third codon positions of the mtCOI full gene and amplicon are completely saturated.

In both phylogenetic trees, the Aleyrodinae subfamily outcompeted the Aleurodicinae subfamily in the number of analyzed species. The Aleurodicinae was represented by species from the genera Paraleurodes and Aleurodus. In both trees, the Aleyrodinae species clustered by genus, four major clusters could be identified and similar clustering pattern was obtained at the genera level (Figure 1 and 2). Some variation between the trees was observed in the orange cluster. In the tree that was based on five Portiera genes, the
Figure 1: BEAST2 inferred Portiera chronogram based on two rRNA (16S and 23S) and three coding genes (groEL, rpoD, and dnaK). Colored branches highlight the four major clades in the Aleyrodinae subfamily. Branch lengths are displayed in Million years. Period, Epoch, and Age are according to the geological time scale standards. Chromohalobacter salexigens DSM3043 was used as outgroup but is not displayed for plotting reasons.

Figure 2: BEAST2 inferred Portiera chronogram based on two rRNA genes (16S and 23S). The sequences were generated in this work and in Thao and Baumann (2004b). Colored branches highlight the four major clades in the Aleyrodinae subfamily. Branch lengths are displayed in Million years. Period, Epoch, and Age are according to the geological time scale standards. Chromohalobacter salexigens DSM3043 was used as outgroup but is not displayed for plotting reasons.
orange cluster was found to be the most basal branch and only contained one species, *T. vaporariorum* (Figure 1). In the tree that was based only on the two rRNA genes, the orange cluster (containing this time five species) was integrated within the purple cluster, and was close to the *Aleyrodes* clade (Figure 2). These topological inconsistencies likely result from the different taxon sampling in the two analyses. The tree that was based on five *Portiera* genes was well supported and most of the nodes presented posterior values greater than 0.9 (Figure 1). In contrast, the tree that was based on two rRNA genes had a large number of nodes with posterior values below 0.8, especially at some inner branches (Figure 1). Some of the low posterior support values were associated with potential species complexes: *B. tabaci*, *Aleyrodes singularis/proletella* and *Neomaskiella andropogonis*. Noticeable, some inconsistencies in taxonomy were also present in both trees. For example, *Aleuroviggianus adanaensis* was almost identical to *Tetraleurodes bicolor* at the sequence level and some species from the genera *Tetraleurodes*, *Trialeurodes*, and *Dialeurodes* were distributed among different clades.

Among the Aleurodicinae subfamily, *Paraleyrodes minei* was the first species to diverge, around 119.68 Mya (Million years ago) (102.74-133.42 95%HPD) or 112.6 Mya (85.49-133.31 95%HPD) according to the five genes- or two genes-based trees, respectively (Figure 1 and 2). The divergence of *Aleurodicus dispersus* from *Aleurodicus flaccissimus* was estimated to be around 20.35 Mya (9.35-33.28 95%HPD) and 30.21 Mya (15.02-47.18 95%HPD) for the five genes- and two genes-based trees, respectively (Figure 1 and 2). These dates were in agreement with previous estimates by Santos-Garcia et al. (2015). In the Aleyrodinae subfamily, despite the topological differences between the two trees, the estimated time of the first cladogenetic event (the first splitting after divergence from the main branch) was similar for the blue, green and purple clusters. The estimated divergence dates for the orange cluster were not comparable between the two datasets. However, if we consider the split between the green and purple/orange clusters in the two rRNA genes-based tree as the origin of the lineage leading to *T. vaporariorum*, then, the estimation for *T. vaporariorum* are quite similar: 97.36 Mya (76.14-116.97 95%HPD) in the two genes-based tree and 110.26 Mya (91.43-126.3 95%HPD) in the five genes-based tree. Again, these estimations were in agreement with previous studies Misof et al. (2014); Santos-Garcia et al. (2015).

The most studied whitelly species, the *B. tabaci* species complex, was part of the green cluster. Our estimations of the emergence time of the *Bemisia* genus and the *B. tabaci* species complex were similar to previous estimations by Santos-Garcia et al. (2015): 44.08 Mya (31.36-57.11 95%HPD) and 7.27 Mya (3.43-11.48 95%HPD) or 47.84 Mya (31.64-64.53 95%HPD) and 11.87 Mya (5.42-19.52 95%HPD), in the five genes- and two genes-based trees. Finally, the divergence between *B. tabaci* species MEAM1 and MED also agreed with previous estimates (Santos-Garcia et al., 2015). Taken together, although topological differences existed between the two trees, the convergence of their divergence time estimates supports their robustness.

**Tracking the origin of genomic instability in Portiera**

*Portiera* from *B. tabaci* lacks the DNA polymerase III proofreading subunit (*dnaQ*). This absence seems to be correlated with the massive rearrangements, large intergenic regions, and repetitive sequences (especially microsatellites) present in this *Portiera* lineage (Sloan and Moran 2013; Santos-Garcia et al. 2015). To locate the evolutionary point in which *Portiera* lost *dnaQ* and genome stability began, we screened our samples for the presence of *dnaQ* and four possible gene order configurations. The two configurations *groEL-rpsA* (A) and *leuC-leuD* (B) are ancient ones and are shared between *Aleurodicus* and *T. vaporariorum*. The other two rearrangements, *lepA-groEL* (A_Bt) and *secA-leuC* (B_Bt), were
found so far only in the *B. tabaci* complex (Figure 3). We were able to amplify *dnaQ* of *Portiera* from all species tested except for *Aleurolobus olvinus*, *Aleurolobus marlatti*, *Singhiella simplex*, *Bemisia afer*, *B. euphorbiarum*, and *B. reyesi*. The first three genera species form a monophyletic clade together with *Bemisia*. Based on our ancestral state reconstruction using the five genes-based tree, it is highly likely (posterior probability of 1) that the most recent common ancestor (MRCA) of this clade also lacked a functional *dnaQ* gene (Fig 3). In addition, when using the two genes-based tree, it is probable that the MRCA of the *Singhiella-Bemisia* clade also lacked a functional *dnaQ* (0.62 posterior probability) (Figure S1). Due to too much uncertainty, we could not resolve the presence/absences of *dnaQ* in deeper nodes. Still, under a maximum parsimony scenario, *dnaQ* is likely to be present in the genome of *Portiera* from all whiteflies, with the exception of the *Singhiella-Bemisia* clade.

*Portiera* of all species outside the *Singhiella-Bemisia* clade also presented the ancestral gene order (rearrangements A and B) (Fig 3). *Portiera* of *Bemisia* species outside the *tabaci* species complex only presented the B<sub>Bt</sub> rearrangement, suggesting a possible different order in region A. Although were unable to recover any transcript containing the B<sub>Bt</sub> region in the *B. tabaci* SSA1 species, this species seems to be syntenic to other *B. tabaci* species (Figure S2). In addition, we could not amplify the ancestral or modified A and B regions in both of the *Aleurolobus* species and *Singhiella simplex*, raising the possibility that several gene rearrangements took place in the A and B regions during the evolution of *Portiera* in the *Singhiella-Bemisia* clade (Fig 3).

![Figure 3: Summary of the screening for the *dnaQ* gene presence or absence and the gene rearrangements. Ancestral state inference was estimated using the five *Portiera* genes-based tree (left). Pie charts at the nodes represent the posterior probability for the presence (blue) or absence (red) of *dnaQ*. Note that all nodes have the probability of 1. The matrix represents the gene rearrangement amplification results (right). The letters above the matrix indicate the four possible rearrangements that were tested. Letters without index refer to the ancestral gene order found in *Portiera* of *Aleurodicus* and *T. vaporariorum*. Letters with the sub-index Bt refer to the gene order found in *Portiera* of *B. tabaci*. Green filled squares represent successful and validated amplifications while gray filled squares indicate that the gene order rearrangements were not tested but the ancestral one (A and B) is assumed. *: full genome available. **: no transcripts containing the B<sub>Bt</sub> region were obtained.](image-url)
The genomic and metabolomic characterization of *Portiera* from *Singhiella simplex*

To further elucidate the origin of *Portiera* genome instability and its putative effects on functionality, we sequenced the genome of *Portiera* from the most basal species in the *Bemisia* clade, the fig whitefly *Singhiella simplex*. As explained in length in the “Materials and Methods” section, the sample also contained individuals of the mulberry whitefly *Pealius mori*, which share some host-plants with *S. simplex*. As we were able to classify and recover complete *Portiera* and mitochondrial genomes from both *S. simplex* and *P. mori*, there was no effect to the accidental mixing on our further analyses. The genome of *Portiera* from *S. simplex* was recovered as 9 contigs (Table 1), all ending in repetitive sequences. It is the largest *Portiera* genome described so far, being 134Kb larger than that of *Portiera* of *B. tabaci* (Table 1). The number of coding genes was similar in *Portiera* of *S. simplex* and *B. tabaci*, which suggests that the genome expansion in *Portiera* of *S. simplex* is due to an increase in the size of the intergenic regions, which account for 40% of the genome. The genome of *Portiera* from *S. simplex* presented the lowest coding density (59.6%) and the highest number of direct (23) and inverted repeats (17) among all analyzed endosymbiont genomes (Table 1). As was already “suspected” from the PCR amplification results, the dnaQ gene was found to be pseudogenized in *Portiera* of *S. simplex*.

Synteny evaluation analysis, based on 235 Orthologous Clusters of Proteins (OCPs) (Figure S3), indicated that *Portiera* of *S. simplex* presents a different genomic architecture when compared both to the ancestral *Poriera* or to *Portiera* of *B. tabaci* gene order (Figure 4A). Still, textitPortiera of *S. simplex* presented a high degree of microsynteny (e.g. operons) with the other *Portiera* genomes. The dnaQ pseudogene is located in a region that has suffered different rearrangements and an expansion of the intergenic regions (Figure 4B). Comparisons to other *Portiera* genomes and different obligatory endosymbionts present in mealybugs, scale insects, and cicadas (Figure S4), indicated that endosymbionts lacking a functional dnaQ present extended intergenic regions (Kruskal-Wallis test, df = 8, p-value < 2.2$^{16}$ and pairwise Wilcoxon test with Benjamini-Hochberg FDR, Figure S4).

Metabolically, the genome of *Portiera* of *S. simplex* is close to *Portiera* of *B. tabaci*. It has incomplete pathways for lysine but can still produce arginine. It has lost the ability to produce tryptophan (the trpF gene is absent) and also lacks the aminoacyl-tRNA synthetases metG and alaS (also lost *Portiera* of *T. vaporariorum*, *P. mori*, and *B. tabaci*) and trpS (lost in *Portiera* of *B. tabaci*) (Table 2). The tRNA$^{lle}$-lysidine synthetase *tilS*, responsible for avoiding miss-charging of methionine instead of isoleucine, was found to be uniquely pseudogenized in *Portiera* of *S. simplex*. In addition, a large gene-loss event (18 genes) occurred in MRCA of the *Singhiella-Bemisia* clade (Table 2 and Figure 4A). This event included the loss of dnaQ and other six genes related to the DNA replication and repair machinery (Table 2).

Comparative Molecular Evolution of *Portiera* lineages

We estimated the ratio of synonymous ($S$) and non-synonymous ($N$) substitutions per site ($dS$ and $dN$) and their omega ratio ($\omega = dN/dS$) in 232 single-copy genes shared between the *Portiera* lineages of six whitefly species: *A. dispersus*, *A. flocchishmus*, *T. vaporariorum*, *P. mori*, *S. simplex* and *B. tabaci* (MEAM1). After filtering, 158 orthologous shared genes were kept. To obtain the $S$ and $N$ per site per year ($dS/t$ and $dN/t$), the values were divided by the lineage divergence time: 19.64 Myr (Million years) for *Aleurodicus*, 111.29 Myr for *Trialeurodes*, 99.98 Myr for *Pealius*, and 71.34 for *Singhiella-Bemisia* (Figure 1). $dS/t$ and $dN/t$
Table 1: General genomic features of Portiera and other endosymbionts lacking dnaQ, presenting large intergenic regions or genome instability.

| Accession Number | Portiera TeVα | Portiera PoMo | Portiera SSi | Portiera BeTa | Uzinura ASNER | Trichlora PVAL | Hodgkinia TETUND1 & TETUNDZ |
|------------------|---------------|--------------|--------------|--------------|---------------|---------------|-----------------------------|
| Genome size (bp) | 3217980 | 3217980 | 3217980 | 3217980 | 3217980 | 3217980 | 3217980 |
| Contigs | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| N50 (bp) | NA | NA | 17038 | NA | NA | NA | NA |
| L50 | NA | NA | 2 | NA | NA | NA | NA |
| GC% | 24.69 | 24.1 | 26.18 | 26.12 | 39.2 | 58.83 | 46.77 |
| Genes* | 307 | 308 | 308 | 284 | 275 | 130 | 177 |
| CDS | 266 | 266 | 252 | 247 | 226 | 116 | 121 |
| Pseudogenes (CDS) | 1 | 1 | 11 | 7 | 13 | 19 | 39 |

*Gene features including pseudogenes. ** as appearing in the annotation. *** 3 of them correspond to the duplicated rRNA operons.

Table 2: Gene losses in Portiera lineages and their MRCA (nodes from Figure 4).

| Species/MRCA | Gene name |
|--------------|-----------|
| A. dispersus (AIDI) | abcC |
| T. vaporariorum (TeVα) | rpmD, purA, abcP, hisE, rplA |
| P. mori (PeMo) | clpP, clpX, lasA, rimM, rsmH |
| S. simplicis (SiSi) | rsmH, abcP, hisA, bts, trfP |

| A | B | C | D | E/F |
|---|---|---|---|---|
| purA, lepB | glyA, hopB, metG, nosA, rnc, tkA, ygfF, alaS | clpP, clpX, lasA, rimM, rsmH | purA, lepB, rpmD, dafP, deaD, dnaL*, dnaQ*, dnaX*, frt, holA*, holB*, hopA*, mucD, mflA*, rsmA, rncC*, trfP, hypothetical protein | clpP, clpX, lasA, ygfH, dafP, era, gldP, sdbB, sdb*, upp |

DNA replication and repair.

are normalized values and allow comparisons between lineages (the branch leading to a specific Portiera genome). For analysis, we used the chronogram that was based on five Portiera genes as it presented higher internal nodes support.

Our analyses indicated that the Portiera lineages evolve at different dS/t (Kruskal-Wallis test, p-value < 2.2e-16) (Figure 5A). Portiera of B. tabaci was the fastest-evolving lineage, followed by Portiera of S. simplicis. Portiera of P. mori was the slowest evolving lineage (Table 3). Also, dN/t values showed statistical differences among Portiera lineages (Kruskal-Wallis test, p-value < 2.2e-16) (Figure 5B). Similar to dS rates, Portiera of B. tabaci and S. simplicis were the fastest evolving lineages while Portiera of P. mori was the slowest evolving lineage (Table 3).

Table 3: Average nucleotide substitutions per site per year and ω ratios for Portiera and mitochondrial lineages.

| Species/MRCA | Lineage | dS/t | dN/t | ω |
|--------------|---------|------|------|---|
| Portiera | A. dispersus (AIDI) | 4.79 x 10^-10 | 2.35 x 10^-10 | 0.1200 |
| T. vaporariorum (TeVα) | 2.48 x 10^-10 | 1.46 x 10^-10 | 0.1490 |
| P. mori (PeMo) | 8.25 x 10^-10 | 1.02 x 10^-10 | 0.2310 |
| S. simplicis (SiSi) | 5.94 x 10^-10 | 4.83 x 10^-10 | 0.0944 |

Mitochondria

| Species/MRCA | Lineage | dS/t | dN/t | ω |
|--------------|---------|------|------|---|
| Trichlora PVAL | A. floccimmus (misAID) | 1.54 x 10^-10 | 2.98 x 10^-10 | 0.0894 |
| T. vaporariorum (TeVα) | 1.05 x 10^-10 | 2.28 x 10^-10 | 0.0576 |
| P. mori (PeMo) | 6.74 x 10^-10 | 2.11 x 10^-10 | 0.2684 |
| S. simplicis (SiSi) | 1.79 x 10^-10 | 3.84 x 10^-10 | 0.2932 |
| B. tabaci (SiSi) | 1.26 x 10^-10 | 3.35 x 10^-10 | 0.0315 |

Next, we tested if the six Portiera lineages differ in the selection forces that act on their genomes by comparing their ω ratios (Figure 5C). Three statistically significant groups were observed (Kruskal-Wallis test, p-value < 2.2e-16): Portiera of B. tabaci and S. simplicis had the lowest ω values, Portiera of A. dispersus and A. floccimmus presented intermediate ω values, and Portiera of T. vaporariorum and P. mori had the highest ω values. Most ω values were close to zero indicating a strong effect of purifying selection.
Figure 4: Portiera genomes syntenic comparisons based on 230 single copy core genes. A) Cladogram summarizing Portiera phylogenetic relationships based on the species tree obtained as part of the OrthoFinder pipeline. Filled circles at the nodes represent the number of coding genes estimated to be present in the most common recent ancestor (MRCA) using COUNT. Filled circles at the leaf tips represent the number of coding genes in each Portiera genome. Letters at the nodes list the MRCA to allow comparisons with Table 2. Portiera genomes are represented linearly with blue boxes representing syntenic genes in the direct strand (upwards) or in the complementary strand (downwards), gray lines connect genes in the same strand, yellow lines connect genes in different strands, twisted lines indicate inversions. The green line highlights the position of functional and non-functional (ψ) dnaQ genes. For Portiera of S. simplex, only contigs containing core genes are represented (seven from nine contigs). B) Magnification of syntenic comparisons between Portiera of P. mori, S. simplex, and B. tabaci AsiaII3.

Selection in almost all tested genes. Still, we detected 18 genes presenting signatures of relaxed/adaptive selection ($dS > 0$, $\omega \geq 1$ & $\omega \leq 10$) in Portiera of T. vaporariorum (9 genes), A. floccissimus (4), P. mori (3), and A. dispersus (1) (Table S4). Some of these genes were found to be related to amino acid biosynthesis ($hisH$, $leuC$, $trpC$, $gatC$), aminoacyl-tRNA synthetases ($sufS$ and $cysS$), or to energy metabolism ($atpB$ and $cyoD$).

Because the absence of host genomic/transcriptomic resources for a sufficient number of whitefly species, we estimated the mitochondrial nucleotide substitution rates as a byproximation of nucleotide substitution rates in the host genomes (Figure 5D-F). Since the mitogenome of A. floccissimus is still not available, we calculated the $dS/t$ and $dN/t$ values of the Aleurodicus lineage using only the mitogenome of A. dispersus (dividing the values by 129.35 Mya, the estimated time when the split between the Aleurodicinae and the Aleyrodinae families occurred). Only 12 genes were included in the analysis since mitogenomes annotation was not consistent.
4 Discussion

**Portiera** as a valuable phylogenetic resource for studying whitefly taxonomy

Unlike taxonomical research in other insect groups that rely on adult morphology for classification, the current taxonomy of whiteflies is mostly based on the morphology of the nymphal stages. These stages present plasticity in many morphological traits that respond to various abiotic and biotic environments including the identity of the plant host (Charles 2010, Manzari and Quicke 2006), eliminating in many cases the possibility of identifying a clear objective criterion. This has led to relatively high number of inaccuracies and mis-assignments in the group taxonomy (Manzari and Quicke 2006). For example, the *Dialeurodes* genus, which is one of the most prolific and studied groups, lacks clear taxonomical keys. As a result, the genus is currently divided into at least five major groups, each containing several genera (Jensen 1999, 2001). Additional support to the current problematic status of whitefly taxonomy comes from two complementing large-scale studies. In the first, an extensive cladistic analysis suggested that around half of the 117 Aleyrodinae genera analyzed were not monophyletic (excluding monobasic genera) (Manzari and Quicke 2006). The second study used puparial morphological characters from all 20 Aleurodicinae genera and DNA sequences from nine Aleurodicinae genera, but recovered only 60% and 14% of the genera as monophyletic, respectively (Charles 2010).

Taking all above in consideration, it is quite safe to state that whitefly taxonomy can significantly benefit from the development of complementary classification frameworks, especially those using molecular data.

The current most popular molecular framework uses *mtCOI* sequences for species “barcoding”. In whiteflies, two *mtCOI*-dependent barcoding approaches are used. The first approach uses the 5' region of the

- **Figure 5**: Synonymous (A) and non-synonymous (B) substitutions per site per year and their ω ratios (C) estimated for 158 core shared genes between Portiera lineages of six whitefly species. Different letters indicate significant statistical differences between lineages (non-parametric Kruskal-Wallis and Wilcoxon post-hoc pairwise tests). Synonymous (D) and non-synonymous (E) substitutions per site per year and ω ratios (F) estimated for 10 full mitochondrial genes from six whiteflies species. N.S. = No significant difference. Different letters indicate significant statistical differences between lineages (one-way ANOVA and Tukey’s post-hoc test).

and COXII was filtered out. The mitochondrial lineages presented similar dS/t and dN/t values (One-way ANOVA, p-value > 0.2) (Figure 5D and E and Table 3). The ω values differed among lineages, being *S. simplex* and *A. dispersus* mitogenomes the ones with the higher and lowest values, respectively (One-way ANOVA, p-value < 0.02 and Tukey’s post-hoc test) (Figure 5F). Yet, in all lineages nearly all ω values were below 0.1, indicating a strong effect of purifying selection. Attending to the dS/t ratio between mitochondria and Portiera, the former is evolving faster. These ratios vary from ten in *B. tabaci* to 100 in *T. vaporariorum*.
mtCOI gene. This region was used by the Consortium for the Barcode of Life (BOLD) initiative (www.barcodeoflife.org) which currently includes 1062 putative species (Barcode Index Number System clusters) and 102 identified species. The second approach, which is traditionally used in Bemisia tabaci research, targets the 3' region of the mtCOI gene (Frohlich et al., 1999; De Barro et al., 2011). Using a modified 5' mtCOI barcoding approach (cocktail of primer sets based on the LCO1490 and HCO2198 primers), the phylogenetic tree published by Dickey et al. (2015) failed to recover the two whitefly subfamily lineages and only few nodes were well supported. Interestingly, the Singhiella-Aleurolobus-Bemisia clade was recovered with reasonable support. Similarly, Ovalle et al. (2014) used the 5' mtCOI to explore the relationship between 8 genera (20 taxa). Again, while deeper nodes were not fully resolved, more recent nodes were well supported. This raises the possibility that the use of mtCOI gene sequences might be a powerful framework for whitefly barcoding (species identification) but a less accurate approach for establishing genus boundaries and genera relationships in the superfamily, as already was proposed for the Aphidoidea (Coeur d’Acier et al. 2014).

The low success of using mitochondria gene sequences for establishing inter-genus relationships in whiteflies, could be an effect of the mitogenome mutation rate, which can be more than 20-times higher that of nuclear genes (Allio et al., 2017). Moreover, whitefly mitogenomes in particular, present several translocations and evolve faster than other hemipteran insects (Thao et al., 2004; Song et al., 2012). Therefore, it is not surprising that we found that many mitochondrial genes show saturation at their synonymous sites, suggesting a compromised phylogenetic signal. For example, the widely used mtCOI gene shows saturation in many third codon positions (Table S3) (Song et al., 2012). Therefore, the third codon position should be excluded from phylogenetic analysis, reducing the effective length of the alignment. Although full mitogenomes can be used to resolve deeper relationships, the mitochondrial genes to be included should be selected carefully to avoid saturated ones. Even so, this approach seems to be unworkable in cases of large screening. A second problem we detected when using the mtCOI universal primers is their relatively low (around 58%) amplification success rate.

The pros and cons of using Portiera gene sequences for studying whitefly taxonomy and phylogenetics

Several studies have proved the accuracy of using gene sequences of primary endosymbionts for reconstructing their host evolutionary history (Martinez-Torres et al., 2001; Jousselin et al., 2009; Nováková et al., 2013; Meseguer et al., 2015; Hall et al., 2016; Meseguer et al., 2017). Here, we found several additional advantages for the use of Portiera gene sequences in inferring the phylogenetic relationships among whiteflies. First, in contrast to mtCOI amplicons, all designed Portiera primers had an almost perfect amplification success except for the rpoD set that failed to amplify one sample. Second, the specific targeting of Portiera genes is by itself a diagnostic tool that allows to differentiate whiteflies from similar insects (e.g., psyllids nymphal stages) and to discriminate between the two whitefly subfamilies. Discrimination is possible because Portiera from the Aleurodicinae subfamily presents two specific insertions in the 23S rRNA gene (Thao and Baumann, 2004b). Third, targeting Portiera genes is especially useful when studying parasitized samples (Table S2), as the use of universal mtCOI primers is in this case problematic. Fourth, Portiera is evolving slower than the mitogenome of whiteflies. Hence, Portiera genes usually do not show phylogenetic signal saturation. This makes the use of Portiera gene sequences more adequate for solving inter-genera relationships and deeper nodes than the mtCOI gene sequence. On the other hand, Portiera gene sequences seem to be limited in their ability to resolve recent speci-
ation events or minor differentiations between cryptic species. It is also likely that at the intra-species level (populations), fast-evolving genes, as the *mtCOI*, will perform better. Last but not least, there are several *Portiera* genomes available, which allows targeting different sets of genes.

**The problematics and complexity of whitefly taxonomy**

Some genera included in our analysis previously presented phylogenetic incongruence. Among them, *Aleurolobus*, *Aleurotrichelus*, *Aleurotrachelus*, *Bemisia*, *Dialeurodes*, *Tetraleurodes*, and *Trialeurodes* had more than one representative in our analysis. Therefore, we tried solving previously observed phylogenetic conflicts by applying the *Portiera* phylogenetic framework established. In the case of *Aleuroplatus*, our results seem to support the paraphyly of these genera. Also, our phylogenetic reconstruction supports the monophyly of the *Dialeurodes* and *Tetraleurodes* genera if *Tetraleurodes bicolor* and the two unidentified *Dialeurodes* samples clustering with *Trialeurodes* are not taken into account. In addition, our analysis confirmed the relationship between *Aleuroclava* and *Dialeurodes*. Based on their early divergence, which fits more a separation time period between genera, both *Dialeurodes citri* and *Dialeurodes hongkongensis* are likely to belong to two different genera, with the latter probably assigned to the *Gigaleurodes* [Jensen 1999; Manzari and Quicke 2006].

*Aleurolobus*, *Aleurotrichelus*, and *Bemisia* were monophyletic in our study and also in previous analysis [Ovalle et al. 2014], in contrast to their paraphyletic status reported in [Manzari and Quicke 2006]. The only *Aleurothrixus* species in our dataset grouped with the *Aleurotrichelus* species, suggesting that this genus could be synonymized with the latter. Although never grouped together [Manzari and Quicke 2006], our work clearly supports the relationship between the *Aleurolobus* and *Bemisia* genera, as was previously suggested [Ovalle et al. 2014]. Interestingly, *Singhiella simplex* established a monophyletic clade together with *Aleurolobus* and *Bemisia* in our work. This finding disagrees with previous studies that suggested relationship between the *Singhiella* and *Dialeurodes* genera [Jensen 2001; Manzari and Quicke 2006]. The possible existence of a *Singhiella-Aleurolobus-Bemisia* clade is also supported by *mtCOI* phylogenetic analysis [Ovalle et al. 2014; Dickey et al. 2015]. However, while morphological classification of *Aleurolobus* species is straightforward [Manzari and Quicke 2006], it could be possible that *Shingella simplex* has been erroneously assigned to the *Shingella* genus.

Several authors have tried to classify the Aleyrodinae into different tribes (reviewed in [Manzari and Quicke 2006]). Despite our limited sampling, our results support some of those classifications. Based on their monophyly and the genome characteristics of their *Portiera* lineage (see below), we propose that the genera *Singhiella*, *Aleurolobus*, and *Bemisia* belong to the Aleurolobini tribe. The Aleurocanthini tribe was suggested to include the *Aleurocanthus*, *Aleurotrichelus*, and *Pentaleurodes* genera. As support for this tribe, we recovered a monophyletic clade including the *Aleurotrichelus* and *Aleurothrixus*. We found that the Neomaskellini tribe includes both the *Neomaskellia* and *Vasdavidius* genera. Two from three *Tetraleurodes* species were recovered as monophyletic, suggesting that the Tetraleurodini tribe is likely to be correct. The tribes Aleyrodini and Dialeurodini are the largest but also the most controversial. In these tribes, we can only confirm the Dialeurodini membership of both *Dialeurodes* and *Aleurolobula*.

Finally, we would like to comment on two inconsistencies in our data. The almost identical *Portiera* sequences of *Aleurovigianus adanaensis*, *Dialeurodes bicolor*, and *Trialeurodes ricini* could be due to
cross-contamination or possible adult miss-classification (e.g. adult near puparias from different species). If Trialeurodes ricini is not considered, our results support the possibility that Trialeurodes is a monophyletic genus (Manzari and Quicke 2006). Contrarily, the identical Portiera sequences of Aleyrodes singularis and Aleyrodes proletella are likely to reflect a recent speciation event with some hosts-plant overlap (Martin et al. 2000). Aleyrodes cross-contamination is unlikely as only Aleyrodes singularis has been found in Israel and gDNA extractions and amplicons amplification were performed separately in time for each species.

Paraleyrodes: a living fossil?

Described at first as Aleyrodes perseae, the nymphal stages of the genus Paraleyrodes present typical Aleurodicinae morphological characters, such as subdorsal compound pores or legs with apical claws (Quaintance 1909). However, adults present morphological characters typical of Aleyrodinae, such as small body size and single-vein wings Quaintance (1909); Martin (1996, 2007). Interestingly, the Paraleyrodes genus presents median ocellus, an ancestral character described in Cretaceous taxa (Drohojowska and Szwedo 2015). Our analysis supports the inclusion of the Paraleyrodes genus inside the Aleurodicinae subfamily based on its ancient origin and the presence of the 23S rRNA insertions common to the Aleurodicinae subfamily (Thao and Baumann 2004b). Our estimates overlap with the calibration point used, suggesting that Paraleyrodes genus originated in the Lower Cretaceous (100.5-145 Mya). Therefore, Paraleyrodes can be considered a long-enduring extant taxon, which may explain the retention of the middle ocellum and the mixture of morphological characteristics of both Aleyrodidae subfamilies. Although speculative, it also could be possible that other hard-to-assign Aleurodicinae genera, such as Aleuroctarthrus (presents medium ocellus) or Palaealeurodicus (does not present clawed legs), are indeed long-enduring taxa (Martin 2008). Using cladistic and molecular analysis, these two genera were closely related to Paraleyrodes (Charles 2010). In addition, Palaealeurodicus was placed as basal to all Aleurodicinae based on four mitochondrial genes (Charles 2010). Therefore, Paraleyrodes can be considered as sister taxon of Palaealeurodicus, which diverged before the radiation of the Aleurodicus genus (Charles 2010). Identifying such kind of long-enduring taxa could be an invaluable resource for understanding the evolution of the whitefly superfamily.

Long-standing endosymbionts present almost static genomes

Adaptation to the intracellular lifestyle has a significant impact on bacterial symbionts. Metabolic redundancy between the host and the endosymbiont promotes the dependency of the later on the former intracellular environment (Morris et al. 2012). Moreover, vertical transmission drastically reduces the endosymbiont effective population size ($N_e$) and the chances to acquire new genetic material, eventually leading to the generation of asexual populations. The combined effects of vertical transmission and intracellular lifestyle promote the accumulation of deleterious mutations that are otherwise pruned by selection in larger $N_e$, which can lead to massive loss of genes (Moran 1996; Toft and Andersson 2010; Wernegreen 2015). The outcome of the process, known as the Muller’s Ratchet (Moran 1996), is an endosymbiont that harbors a highly reduced genome, with small intergenic regions and very few repetitive sequences (Toft and Andersson 2010; Wernegreen 2015). Common conserved elements include genes that are essential for producing the host requirements and a minimal informational and translational machinery required for cell maintenance (Moran and Bennett 2014).

As a consequence of the reduced or absent replication and recombination machinery, and
the minimal presence of repetitive sequences, long-standing endosymbionts present almost static genomes (Moran and Bennett, 2014). For example, only few inversions were detected in endosymbionts that have been co-diverging with their host for more than 100 Myr (Patiño-Navarrete et al., 2013; Chong et al., 2019). *Portiera* of whiteflies is not different from other long-standing endosymbionts and usually harbor “classical” reduced and static genomes (Sloan and Moran, 2013; Santos-Garcia et al., 2015). One exception to this “rule” is *Portiera* of *B. tabaci*, which presents a genome with large intergenic regions, extensive rearrangements, and abundance of repetitive sequences (Sloan and Moran, 2013; Moran and Bennett, 2014; Santos-Garcia et al., 2015).

### Genome instability in *Portiera* of the tribe Aleurilobini

The genome of *Portiera* from *B. tabaci* presents one of the most reduced sets of DNA replication and repair genes among known long-standing P-endosymbionts, including the loss of the dnaQ gene which encodes the DNA polymerase proofreading subunit (Moran and Bennett, 2014). This loss has been linked to the uncommon extensive genome rearrangements, inversions, abundance of repeated sequences, large intergenic regions, and accelerated evolution found (Sloan and Moran, 2013; Santos-Garcia et al., 2015). Our findings here suggest that the massive loss of DNA replication and repair genes is not restricted to *B. tabaci*, but shared by all other members of the *Singhiella-Aleurilobus-Bemisia* clade (hereafter the tribe Aleurilobini for simplicity). Therefore, it is quite probable that dnaQ was pseudogenized in the last common ancestors of the tribe, more than 70 Mya.

So far, only three genomes displaying long intergenic regions, genome instability, or the lack of functional dnaQ have been sequenced from other long-standing endosymbionts: *Uzinura diaspidicola*, *Tremblaya princeps*, and *Hodgkinia cicadicola* (Moran and Bennett, 2014; Van Leuven et al., 2014; López-Madrigal et al., 2015; Lukasik et al., 2018). Only a single genome of *U. diaspicola* is currently available, and therefore, it is not clear if this endosymbiont which lacks dnaQ also displays significant genome instability. Relative to the genomes of *Portiera* from *B. tabaci* and *S. simplex*, the genome of *U. diaspicola* presents lower number of repeated sequences and smaller intergenic regions. One explanation to this could be the conservation of the mutL gene in *U. diaspicola* (Moran and Bennett, 2014). The enzyme MutL, together with MutS, is part of the mismatch repair system that corrects mismatch events that are produced by base miss-incorporation and polymerase slippage (Rocha, 2003). The genome of *Tr. princeps* presents genome instability signatures such as long intergenic regions, gene conversions, and the presence of several direct/indirect sequence repeats (López-Madrigal et al., 2015). Still, the number of repeats and the length of the intergenic regions are smaller than in *Portiera* of *B. tabaci* or *S. simplex*. The inactivation of the recombination machinery in *Tr. princeps* has been proposed as a strategy to reduce the number of homologous recombination events and their deleterious consequences in highly reduced genomes (López-Madrigal et al., 2015). However, *Tr. princeps* has access to a complementing recombination machinery as it harbors the endosymbiont *Moranella endobia* which has an active recombination machinery (López-Madrigal et al., 2015). The presence of a functional dnaQ subunit in *Tr. princeps* and the possible access to a complementing recombination machinery suggest that the possible causes of genome instability in *Tr. princeps* are different from that of *Portiera*.

One of the most extreme cases of genome instability was reported in *H. cicadicola*. In some cicada genera, which usually have a long lifespan, *H. cicadicola* has been split into several lineages with different genomic content within the same insect. This enforces functional complementation between the
lineages for normal growth (Van Leuven et al., 2014; Lukasik et al., 2018). Although the genomic architecture of *H. cicadicola* resembles that of *Portiera* presenting unstable genomes, there are major differences in their relationship with their hosts. While *Portiera* is essential for whiteflies, *H. cicadicola* is a co-primary endosymbiont in cicadas and has been replaced several times (Lukasik et al., 2018; Matsuura et al., 2018). Therefore, the selection forces acting on both endosymbionts could be very different: strong purifying selection in the case of *Portiera* while more relaxed, or even non-adaptive selection in the case of *H. cicadicola* (Lukasik et al., 2018).

Large intergenic regions can allow *Portiera* to better tolerate rearrangements while the expansion of repeated sequences can increase the chance of deleterious homologous recombination events (Sloan and Moran, 2013). Considering that the *Portiera* genome presents signs of gene conversion and recombination, it could be possible that long intergenic regions and intergenic repeats are selected in *Portiera* presenting unstable genomes to increase their resilience against deleterious mutations. Repeated sequences mostly accumulate at the intergenic regions and pseudogenes of *Portiera* from *B. tabaci* and *S. simplex* suggesting strong purifying selection at the gene level. However, it could be possible that recombination is also counter-selected in *Portiera* with unstable genomes. This could be the reason why *Portiera* lineages within the *B. tabaci* species complex are syntenic after, at least, 7 My of divergence and present low number of direct/indirect repeats compared to *S. simplex*. In the later, recombination seems still active. Therefore, it could be possible that after a period of genome instability and intergenic regions expansion, direct and indirect repeats are counter selected to favor more stable genomes.

In contrast, the location of tandem repeats in *Portiera* of *T. vaporariorum* (8 over 10) and *P. mori* (24 from 31) partially or completely overlap with coding genes. As sequence repeats in coding genes can cause gene inactivation and/or re-arrangements, their existence within genes of stable *Portiera* genomes can reflect the presence of a minimal, but functional, DNA repair machinery that allows a more relaxed purifying selection process. In fact, *Portiera* of *B. tabaci* and *S. simplex* showed the lowest ω values, indicating stronger purifying selection forces acting on their genomes. Taking together, it could be possible that increased resilience combined with a strong purifying selection force at the gene level, have helped to maintain *Portiera* away from extinction in the Aleurilobini tribe (Bennett and Moran, 2015).

**Possible processes affecting substitution rates in *Portiera***

There are clear differences in the substitution rates among *Portiera* lineages presenting a relatively similar set of recombination and repair genes. *Portiera* of *B. tabaci* substitution rates are close to those of two known accelerated endosymbionts: *Blochmania* (P-endosymbiont of ants) and *Buchnera* (P-endosymbiont of aphids) (Silva and Santos-Garcia, 2015). The substitution rates of *Portiera* from *S. simplex* fall in the range of free-living bacteria, as previously reported for *Portiera* of *Aleurodicus* and *Trialeurodes* (Santos-Garcia et al., 2015). *Portiera* of *P. mori* has substitution rates smaller than free-living bacteria and closer to those estimated for *Sulcia* (P-endosymbiont of cicadas), one of the oldest and slow-evolving endosymbiotic lineages described so far (Silva and Santos-Garcia, 2015).

One possible factor that can have an effect on the mutation rates of endosymbionts is their host generation time (Santos-Garcia et al., 2015; Silva and Santos-Garcia, 2015). There seems to be a positive correlation between the number of generations produced by the insect host and the *Portiera* substitution rates. For example, while *A. dispersus*, *A. floccissimus*, *T. vaporariorum*, and *B. tabaci* establish multiple generations nearly all year
round, *S. simplex* and *P. mori* produce only around four ∼generations per year (Table S2).

A second factor is related to the endosymbiont generation time (Santos-Garcia et al., 2015; Silva and Santos-Garcia, 2015). It has been reported that increased mutation rates and genome reduction are negatively correlated with cell growth (Nishimura et al., 2017). This means that differences in gene losses can result in reduced metabolism in some lineages leading to slower growth rates. In the case of *Portiera* from *B. tabaci* and *S. simplex*, their gene content is more reduced than the rest of the *Portiera* lineages, despite having larger genomes. Moreover, in these two *Portiera* lineages, several gene losses (*dnaX*, *dnaN*, and *holAB*) are likely to affect the DNA polymerase III holoenzyme processivity. Longer replication time could produce slow-growing *Portiera* phenotypes, hence, longer generation times. Additionally, *Portiera* from *B. tabaci* has lost *ssb*, which plays a central role in DNA replication/repair (Shereda et al., 2008), increasing both the mutation rates and generation time compared to *Portiera* of *S. simplex*. It seems, therefore, that the increase in generation time can result both in reduced and increased mutational rates, depending on the mechanism involved. Reduced metabolic performance and longer replication time will decrease the mutational bias by restricting the number of *Portiera* cells divisions (Santos-Garcia et al., 2015; Silva and Santos-Garcia, 2015). On the other hand, increasing the replication time in the cell cycle due to reduced processivity of DNA polymerase III or reduced/lack of functionality of other important component in the replication machinery will promote higher mutation rates in spite of the prolonged generation time (Nishimura et al., 2017).

Another factor that could affect the substitution rate of *Portiera* genomes is the possible complementation of the reduced DNA replication and repair machinery by host nuclear-encoded proteins. The host may exert this by two non-exclusive mechanisms. The first is using bacterial genes that have been horizontally transferred to the host nuclei and are targeted back to the *Portiera* cell. The second involves the re-utilization of host mitochondria-targeted proteins (Santos-Garcia et al., 2014, 2015; Silva and Santos-Garcia, 2015). Although highly speculative, it is possible for the host cell to re-target the mitochondrial polγ, which presents proof-reading activity, towards *Portiera*. Since *mutS* is present in all *Portiera* genomes, a combination of imported proteins and MutL-independent mismatch repair mechanism (Martí et al., 2002) can help to reduce the mutation rate in *Portiera* lineages, especially those presenting a reduced recombination and repair machinery. The possible involvement of host complementation mechanism is also supported by the finding that some host DNA replication and repair genes and several aminoacyl tRNA synthetases are over-expressed in *B. tabaci*’s bacteriocyte compared to the rest of the body (Mao et al., 2018).

**The symbiont or the egg: genome instability and bacteriocyte inheritance**

Since the beginning of the research on insect symbiosis, it was clear that the whitefly superfamily presents a special mode of symbiont transmissio as whole maternal bacteriocytes are inherited by the offspring (Buchner, 1965). In *T. vaporariorum*, *Aleyrodes proletella*, *Aleurodes aceris*, and *Aleurochiton aceris* several bacteriocytes migrate to the oocyte (from five to ten depending on the species), entering through the future pedicel (Tremblay, 1959; Buchner, 1965; Szklarzewicz and Moskal, 2001). In contrast, in *B. tabaci*, *Bemisia aff. gigante*, and *Aleurolobus olivinus*, a single bacteriocyte enters the oocyte (Tremblay, 1959; Buchner, 1965; Coombs et al., 2007).

We are aware that the phylogenetic relationships of *B. aff. gigantea* are not completely resolved, but it does seem to be a sister clade of *Aleurolobus* and *B. afer* and distantly re-
lated to *B. tabaci* (Manzari and Quicke, 2006). Therefore, according to the current published data, all the whitefly species that present a single-bacteriocyte mode of inheritance belong to only one phylogenetic group, the tribe Aleurilobini. The most parsimonious explanation suggests that the single-bacteriocyte mode of inheritance has evolved in the common ancestor of *Aleurolobus-Bemisia*, otherwise we will have to assume that it evolved multiple times in different species: *Aleurolobus olivinus, B. aff. gigantea* and *B. tabaci* (Tremblay, 1959; Coombs et al., 2007; Luan et al., 2016, 2018; Xu et al., 2019). Although we lack information on the mode of transmission of the bacteriocyte in *S. simplex*, there is a possibility that this species also presents the single-bacteriocyte mode of inheritance. If found to be true, it would suggest that the whole Aleurilobini tribe is likely to present this derived type of bacteriocyte inheritance.

There is an apparent relationship between the emergence of a single-bacteriocyte inheritance mode and the presence of *Portiera* lineages that show genomic instability. The evolution of a single-bacteriocyte inheritance mode could have had a big impact on *Portiera* evolution since it decreases the effective population size ($N_e$) drastically compared to multi-bacteriocyte inheritance. The extremely low $N_e$ probably intensified the effect of random genetic drift and accelerated the accumulation of deleterious mutations in *Portiera*. In addition, all the *Portiera* cells that are harbored in the same bacteriocyte are expected to present a homogenized allelic composition since recombination events, if happen, are limited to the cells inhabiting the same bacteriocyte. This implies a low chance of recovery of deleterious allele forms once they are established.

At the same time, the single-bacteriocyte inheritance mode also exerts strong purifying selection at both the bacteriocyte and *Portiera* levels each generation as offspring harboring a bacteriocyte or *Portiera* with deleterious mutations will probably suffer from severe fitness costs (Luan et al., 2018). This is somewhat supported by the evidence that extant *Portiera* of the tribe Aleurilobini present moreover a stable gene content, with the massive gene loss events occurring only in their common ancestor. For instance, after approximately 70 Myr of divergence, only five and ten genes were lost from the *Portiera* genomes of *S. simplex* and *B. tabaci*, respectively.

Further research on the tribe Aleurilobini is required in order to determine what occurred first: the transition from the multi- to the single-bacteriocyte inheritance mode or the switch from stable to unstable genomic architecture of *Portiera*. In the first case, the evolution of a different mode of transmission could have triggered the DNA replication and repair machinery loss as purifying selection was not able to maintain them under very low $N_e$ (Lynch, 2010). In addition, these losses may have been complemented by an overtake of some of their activities by the genome of the host cell (Santos-Garcia et al., 2014, 2015; Silva and Santos-Garcia, 2015; Mao et al., 2018). In the latter case, we should assume that *Portiera* lost their recombination and repair machinery as a consequence of a continuous genome degradation process (Bennett and Moran, 2015). This increased the chances for transmitting *Portiera* with deleterious mutations. A multiple-bacteriocytes inheritance mode results in the transmission of mixtures that can mask the presence of bacteriocytes harboring *Portiera* with deleterious mutations/variations. Instead, if single bacteriocytes are inherited, the *Portiera* presenting deleterious mutations will reduce the fitness of the new-born carrying them and they will be counter-selected. Therefore, the evolution of the single-bacteriocyte inheritance mode could have been a compensatory adaptation mechanism of the insect host to exercise an iron grip over *Portiera* transmission for ensuring the viability of its offspring (Campbell et al., 2018).
5 Conclusion

We argue here that the higher classification of whiteflies, at the levels of genera or tribe, requires reassessment. This problem is especially evident in the Aleyrodinae subfamily, which is highly diverse. The current use of nymph morphological characters is not sufficient, and molecular tools must be developed to support the classification of many species/genera in the superfamily. Our work brings evidence that the sequences of target genes of the primary endosymbiont of whiteflies, Candidatus Portiera aleyrodidarum, are a promising phylogenetic resource. They can be used to establish inter-genera relationships, can serve as diagnostic tools by themselves, and can help in the classification of problematic samples (even parasitized ones).

The ability of the established phylogenetic framework to solve standing inconsistencies in whitefly taxonomy also helped us localize a critical event in Portiera evolution, the appearance of genomic instability, which is very uncommon in primary endosymbionts. Our analyses suggest that the Portiera ancestor of the Aleurolibini tribe suffered a massive DNA replication and repair genes loss. This event was the trigger of the genomic instability shown by all extant Portiera lineages in the tribe. We hypothesize that the appearance of genomic instability is also related to the evolutionary switch made between multi- and single-bacteriocyte mode of inheritance.

6 Data deposition

Supplementary Material and Methods, Figures and Tables can be found at XXXX. Relevant scripts and files generated in this work will be available at https://figshare.com/s/8c7c9b4b1db43749ab6e. Sanger sequenced Portiera and mtCOI genes, Novaseq raw reads, and Portiera and mitochondrial assembled genomes have been deposited at the European Nucleotide Archive (ENA) under the project number PRJEB31657 and will be released after acceptance for publication.

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8 Author contribution

DS-G and SM conceived the study. DS-G performed bioinformatics analysis and collected whitefly samples. NM-R performed molecular work and collected whitefly samples. DO supplied NHM whitefly samples and helped in whiteflies taxonomy. DS-G drafted the manuscript with inputs from SM. EZ-F and DO reviewed and corrected advanced versions of the manuscript.
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