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Authors
van Ingen-Buijs, Valerie A
van Westerhoven, Anouk C
Skiadas, Petros
et al.

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Regular Articles

Phyllosticta paracitricarpa is synonymous with the EU quarantine fungus *P. citricarpa* based on phylogenomic analyses

Valerie A. van Ingen-Buijs [a,b,c], Anouk C. van Westerhoven [b,d], Petros Skiadas [d], Xander C.L. Zuijdgeest [e], Sajeet Haridas [f], Christopher Daum [f], Kecia Duffy [f], Jie Guo [f], Hope Hundley [f], Kurt LaButti [f], Anna Lipzen [f], Jasmyn Pangilinan [f], Robert Riley [f], Jie Wang [f], Mi Yan [f], Francis Martin [g], Kerrie Barry [f], Igor V. Grigoriev [f,h], Johannes Z. Groenewald [a], Pedro W. Crous [a,b], Michael F. Seidl [d,*]

[a] *Evolutionary Phytopathology group, Westerdijk Fungal Biodiversity Institute, Utrecht 3584 CT, the Netherlands*
[b] *Department of Plant Sciences, Laboratory of Phytopathology, Wageningen University and Research, Wageningen 6708 PB, the Netherlands*
[c] *Netherlands Institute for Vectors, Invasive plants and Plant Health (NIVIP), National Plant Protection Organization (NPPO), Netherlands Food and Consumer Product Safety Authority (NVWA), Geertjesweg 15, 6706 EA, Wageningen, the Netherlands*
[d] *Theoretical Biology & Bioinformatics, Utrecht University, Utrecht 3584 CH, the Netherlands*
[e] *Centre for Plant Molecular Biology, University of Tübingen, 72076 Tübingen, Germany*
[f] *US Department of Energy Joint Genome Institute, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA*
[g] *Department of Biology, Institut National de la Recherche Agronomique, UMR INRA-Université de Lorraine "Interaction Arbres/Microorganismes", Champenoux F-54280, France*
[h] *Department of Plant and Microbial Biology, University of California Berkeley, CA 94720, USA*

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ABSTRACT

Phyllosticta citricarpa is an important citrus-pathogen and a quarantine organism in the European Union. Its recently described relative, *P. paracitricarpa*, is very closely related and not listed as a quarantine organism. *P. paracitricarpa* is very difficult to distinguish from *P. citricarpa*, since its morphological features overlap and the barcoding gene sequences that were originally used to delimit them as distinct species have a low number of species-specific polymorphisms that have subsequently been shown to overlap between the two clades. Therefore, we performed extensive genomic analyses to determine whether the genetic variation between *P. citricarpa* and *P. paracitricarpa* strains should be considered to represent infraspecific variation within *P. citricarpa*, or whether it is indicative of distinct species. Using a phylogenomic analysis with 3,000 single copy ortholog genes and whole-genome comparisons, we determined that the variation between *P. citricarpa* and *P. paracitricarpa* can be considered as infraspecies variation within *P. citricarpa*. We also determined the level of variation in mitochondrial assemblies of several *Phyllosticta* species and concluded there are only minimal differences between the assemblies of *P. citricarpa* and *P. paracitricarpa*. Thus, using several orthogonal approaches, we here demonstrate that variation within the nuclear and mitochondrial genomes of other *Phyllosticta* species is larger than variation between genomes obtained from *P. citricarpa* and *P. paracitricarpa* strains. Thus, *P. citricarpa* and *P. paracitricarpa* should be considered as conspecific.

1. Introduction

Citrus fruits are popular worldwide and have a multitude of uses and applications. *Citrus* species are however threatened by plant pathogens that may severely limit or even destroy harvests, resulting in great economic losses. One of the most serious pathogens of *Citrus* is *Phyllosticta citricarpa*, the fungus that causes Citrus Black Spot (CBS; Kotzé, 2000). CBS was originally described from Australia, where it causes an estimated ~ 80 M AUD in economic losses annually (Drenth, 2018). It has subsequently spread to nearly all citrus growing areas in the world (CABI, 2022), where it may cause severe crop losses. For instance, in Ghana, CBS was reported to be responsible for up to 22 %

* Corresponding author.
E-mail address: m.f.seidl@uu.nl (M.F. Seidl).

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crop losses (Brentu et al., 2012). Typical symptoms include small and dark sunken lesions that may develop pycnidial conidiomata on fruit, and necrotic spots with a light centre and darker edge on leaves. Atypical symptoms such as virulent spot and lacy spot may also occur infrequently (Miles et al., 2019; Truter, 2010). *Phyllosticta citricarpa* can colonize and cause disease on a broad range of *Citrus* species such as *C. limon, C. sinensis,* and *C. reticulata* (EPPO, 2020; Miles et al., 2019). *Phyllosticta citricarpa* is a quarantine organism in the European Union, which means that if even one fruit contains CBS symptoms, an entire shipment containing up to thousands of fruits is rejected, potentially with severe economic consequences for the exporting company and country of origin.

Phyllosticta citricarpa is present worldwide, but is considered absent from Europe by EPPO (European Plant Protection Organisation) and CABI (Centre for Agriculture and Bioscience International, 2022; EPPO, 2020; Guarnaccia et al., 2017). However, Guarnaccia et al. (2017) collected material from 95 different sites throughout citrus-growing areas in Europe, and found 20 isolates in three countries (Italy, Malta, and Portugal) representing two genetically distinct clonal populations, suggesting two separate introduction events of *P. citricarpa* into Europe, yet no CBS symptoms were observed. As stated by EFSA (The European Food Safety Authority, Jeger et al., 2018), later surveys conducted by the respective countries' NPPOs confirmed the absence of CBS symptoms. The suitability of the Mediterranean climate for development of CBS symptoms is a much-debated subject with no apparent conclusive answer (Baker et al., 2009; Er et al., 2013; Fourie et al., 2017; Martínez-Minaya et al., 2017, 2015; Paul et al., 2005). Nonetheless, a recent outbreak of CBS in Tunisia and further climatic modelling based on data from this outbreak of CBS in Tunisia seems to indicate that the Mediterranean climate may be suitable during specific conditions that may rarely present themselves (EPPO, 2019; Galvañ et al., 2022). Thus, the presence of *P. citricarpa* in Europe remains uncertain.

Phyllosticta is a large genus with more than 3,000 names listed, and species in this genus can occur as endophytes, saprophytes or plant pathogens (Index Fungorum, n.d; Wikee et al., 2013b). In 2002, the genus was revised by Van der Aa and Vanev, who accepted 141 different species colonizing a broad range of plant hosts (Index Fungorum, n.d; van der Aa and Vanev, 2002; Wikee et al., 2013b). *Phyllosticta* contains several species that can colonize *Citrus*. This includes the pathogen *Phyllosticta citriasiana*, which is mainly found on *Citrus maxima* (Wulandari et al., 2009). *Phyllosticta citrichinaensis* has been described as a weak pathogen of several *Citrus* species, but since Koch's postulates have never been demonstrated, it is uncertain to what extent this species can cause disease (Wang et al., 2012). In an attempt to elucidate its lifestyle, we recently performed genomic analyses and concluded that its genome shares attributes with both endophytes and pathogens, suggesting that it has an intermediate lifestyle (Buijs et al. 2022). *Phyllosticta citribraziliensis* is an endophyte described from *Citrus limon* in Brazil (Glienke et al., 2011). Lastly, *Phyllosticta capitalensis* can be found on many plant hosts, and although it is found in *Citrus* as an endophyte, it may cause disease on other hosts such as orchids (Silva et al., 2008; Wikee et al., 2013a).

Phyllosticta paracitricarpa was recently described from *Citrus*, and is morphologically and genetically highly similar to *P. citricarpa* (EPPO, 2020; Guarnaccia et al., 2017). For example, conidiophores of *P. paracitricarpa* are longer and slightly narrower, and conidiogenous cells and conidia are larger than those of *P. citricarpa* (Guarnaccia et al. 2017); conidial sizes are (9-)11–13(−15) x 7–8(−9) for *P. paracitricarpa* versus (10−)11–12(−14) x 7(−8) for *P. citricarpa*. Thus, although the averages are higher, the range shows considerable overlap, which could represent infraspecific variation (Guarnaccia et al., 2017; van der AA, 1973). In addition, *P. paracitricarpa* strains produce a yellow pigment on MEA, while *P. citricarpa* strains do not (Guarnaccia et al. 2017).

As *P. citricarpa* has a quarantine status, and *P. paracitricarpa* currently does not, the ability to indisputably distinguish the two species is of great importance, especially for the National Plant Protection Organisations that are tasked with performing phytosanitary border controls at the European Union borders. Molecularly, the two phylogenetic clades were originally distinguished based on partial DNA sequence data of the *tef1* gene, and to a much lesser extent the partial 28S nrRNA (LSU) gene (Guarnaccia et al., 2017). A method to distinguish *P. citricarpa* from *P. paracitricarpa* based on three SNPs and two indels in the *tef1* sequence was recently proposed (Zajc et al., 2023). However, Ioos *et al.* showed that some strains that displayed *tef1* sequence polymorphisms, which are considered to be typical for *P. paracitricarpa*, clustered with *P. citricarpa* in their phylogenetic analyses when more genes (51) were used (Ioos et al., 2023). In addition, *P. paracitricarpa tef1* sequences contained infraspecific polymorphisms. Together, these data demonstrate that *tef1* is not a good genetic marker to distinguish these two clades. Furthermore, the authors assigned strains into separate clades using a phylogenetic analysis based on 51 single-copy genes, and performed a microsatellite analysis on a subset of the strains to test their method. Of the 51 genes, only 14 (27.5 %) were polymorphic and useful to distinguish between *P. citricarpa* and *P. paracitricarpa* strains, 28 genes (54.9 %) were identical between the two species, and nine genes (17.6 %) exhibited polymorphisms that were not specific to *P. citricarpa* or *P. paracitricarpa*. Based on this separation, a genomic region that allows for distinction of two clades has been identified and subsequently a new qPCR-based marker to distinguish *P. citricarpa* and *P. paracitricarpa* has been developed. The authors described the polymorphisms in this distinctive genomic region as two 7-bp insertions/deletions and two SNPs. However, upon closer inspection the variation in this region appears to consist of a single 35-bp inversion (from base 516,826 to and including 516,860 on scaffold_23). Although useful for distinction of the clades, this variation thus appears to be the result of a single mutation event, indicating a close relation of the two clades. These data do therefore not resolve whether these clades should be considered as one species or two.

Phyllosticta paracitricarpa was described from leaf litter in one *Citrus limon* orchard in Greece (Guarnaccia et al., 2017). However, Wang et al. (2012) reported that *P. citricarpa* strains isolated from spots on *Citrus sinensis* fruits grouped into two subclades, and sequence data from two of their strains from "subclade II" were included in the study by Guarnaccia et al. (2017). As these strains grouped with *P. paracitricarpa*, all strains in this subclade (a total of 16) could also be considered as *P. paracitricarpa*. However, as the identity of these strains is based on *tef1* sequence data, which has subsequently been shown to be unreliable (Ioos et al., 2023), these strains could also represent *P. citricarpa*. In addition, several *P. paracitricarpa* strains isolated from symptomatic fruit were recently reported from China by both Guarnaccia et al. (2017) and Wang et al. (2023). However, there are some inconsistencies as to the identity of the Chinese *P. citricarpa / P. paracitricarpa* strains from Wang et al., as some of the *P. paracitricarpa* strains grouped within the *P. citricarpa* clade based on their *tef1* sequence phylogeny (suppl. fig. S4 in Wang et al. 2023), suggesting that these are in fact not *P. paracitricarpa* strains, at least based on the *tef1* species definition. Lastly, Ioos et al. (2023) reported one new *P. paracitricarpa* strain from China and one from Bangladesh, the latter of which could also be considered *P. citricarpa* based on the *tef1* sequence. In conclusion, a limited number of *P. paracitricarpa* strains have been reported and because nearly all of their identifications as *P. paracitricarpa* are based on *tef1* sequence data, it is uncertain whether these strains are actually belonging to *P. paracitricarpa* and not in fact to *P. citricarpa*.

With such closely related clades, one cannot help but question whether they should be regarded as separate species. The original species delimitation was largely based on differences in the *tef1* gene sequence, which has now been shown not to hold up as species-specific with larger sampling (Ioos et al., 2023). Generally, species delimitation for fungi is based mostly on either morphological features and/or DNA barcoding genes. As *P. citricarpa* and *P. paracitricarpa* are nearly identical in these aspects, another method is required. One approach to determine whether these strains are conspecific, is to study their genomes and

those of closely related species. Ioos et al. (2023) showed that, based on their analyses using 51 concatenated genes (84,155-bp alignment), average percentage relatedness was extremely high at 99.26 % (35 species-specific polymorphic sites), suggesting the two clades might be lineages within one species rather than two separate species. However, further genomic analysis is necessary to provide more information on infra- and inter-species relatedness and to aid in putting these data in perspective, as has been done for species of *Alternaria* (Dettman and Eggertson, 2021). If one were to describe the genomic variation that is present within other well identified and accepted species within the genus *Phyllosticta*, it should be possible to determine whether the variation between *P. citricarpa* and *P. paracitricarpa* should be considered as infra- or interspecific variation. Until now, no analysis of infraspecific genomic variation of citrus-colonizing *Phyllosticta* species has been performed. In this study, we make use of 3,000 single copy ortholog genes to generate a phylogenomic tree. Next, we also generated whole-genome comparisons to determine genomic variation within *Phyllosticta* species. Lastly, we determined how much variation is present in the mitochondrial assemblies of these species. Thus, in this study, we performed an extensive analysis to determine the levels of variation within and between species of *Phyllosticta*, and we used these data to determine whether *P. paracitricarpa* should be considered as synonym of *P. citricarpa*.

2. Materials and methods

2.1. Genome sequencing

For this study, 16 genomes were sequenced and assembled, and seven previously published genomes available at the time (June 2022) and sequenced with similar technology, were also used. The total number of genomes per species used for the majority of analyses in this study is six for *P. capitalensis*, three for *P. citriasiana*, two for *P. citribraziliensis*, eight for *P. citricarpa*, two for *P. citrichinaensis*, and two for *P. paracitricarpa*. All information on the genomes can be accessed at MycoCosm: https://mycocosm.jgi.doe.gov/Phyllosticta. The JGI database identifiers (DBIDs), collection numbers, genome size and BUSCO scores can also be found in Suppl. Table S1. For the 12 gene-region analyses, two additional, previously published, *P. paracitricarpa* genomes were used. DNA isolation was performed as described previously (Buijs et al., 2022). In short, liquid cultures (250 mL Malt peptone broth) were grown for 10–14 days, after which DNA was isolated using the Qiagen Genomic-tip 100/G kit and the Qiagen Genomic DNA buffer set. Library preparation was performed in one of two ways: either 5 ug genomic DNA was sheared to > 10 kb using Covaris g-tubes, or to 15–20 kb using the Megaruptor 3 (Diagenode). The sheared DNA was treated with exonuclease to remove single-stranded ends and DNA damage repair mix followed by end repair and ligation of blunt adapters using SMRTbell Template Prep Kit 1.0 (Pacific Biosciences) or SMRTbell Express Template Prep Kit 2.0 (PacBio). The libraries were purified with AMPure PB Beads (PacBio). PacBio Sequencing primer was then annealed to the SMRTbell template library and sequencing polymerase was bound to them using the Sequel II Binding kit 1.0. The prepared SMRTbell template libraries were then sequenced on a Pacific Biosciences' Sequel II sequencer using 8 M v1 SMRT cells and Version 1.0 sequencing chemistry with 1x900 sequencing movie run times. Assembly was performed using either Falcon (Chin et al., 2016) or Flye (Kolmogorov et al., 2019). For more details on assembly, and on which approach was used for which genome specifically, see Suppl. Table S2. Annotations were performed using the JGI fungal annotation pipeline (Grigoriev et al., 2014). Quality assessment was performed using Quast v5.2 (Gurevich et al., 2013) to assess parameters such as GC content and number of contigs, and BUSCO v5.4.2 (Manni et al., 2021) was used to assess genome completeness using the *Dothideomycetes* odb10 dataset and otherwise default parameters.

2.2. Phylogenetic analyses using ten barcoding genes

To assess if the newly sequenced genomes challenge the established phylogeny of *Phyllosticta* species, we generated a phylogenetic tree using the sequences of 12 full-length barcoding genes of which partial gene sequences are commonly used in molecular phylogenetic analyses of diverse groups of fungi (Crous et al., 2021). The complete gene sequences (nucleotide) of ten protein-coding barcode marker loci as well as two regions of the nuclear ribosomal RNA gene operon, namely the internal transcribed spacers and intervening 5.8S nrDNA (ITS) and the nuclear large subunit nrDNA (28S; LSU) were retrieved from the 23 *Phyllosticta* genomes above as well as two Chinese *P. paracitricarpa* strains that were published in 2023. The LSU of *P. citriasiana* CBS 120486 (Phycit1) was missing from the genome assemblies and could therefore not be included in the analyses. The genome of *Botryosphaeria dothidea* was used as outgroup (JGI identifier Botdo1_1). The protein-coding loci are: actin gene (*actA*), calmodulin gene (*CaM*), chitin-synthase 1 gene (*chs1*), glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*), histone H3 gene (*his3*), DNA replication licensing factor (*mcm7*), DNA-directed RNA polymerase II largest subunit gene (*rpb1*), DNA-directed RNA polymerase II second largest subunit gene (*rpb2*), translation elongation factor 1-alpha gene (*tef1*), and beta-tubulin gene (*tub2*). The sequences were individually aligned using the online interface of MAFFT v7 (https://mafft.cbrc.jp/alignment/server/index.html; Katoh et al., 2019; Kuraku et al., 2013), after which they were concatenated using SequenceMatrix v1.9 (Vaidya et al., 2011). The concatenated alignment consisted of the 25 *Phyllosticta* isolates and the outgroup taxon and the partitions were as follows: *CaM*: 1–1,040; *actA*: 1,041–2,736; *chs1*: 2,737–6,053; *gapdh*: 6,054–7,529; *his3*: 7,530–8,160; *mcm7*: 8,161–10,850; *rpb1*: 10,851–16,439; *rpb2*: 16,440–20,329; *tef1*: 20,330–22,502; *tub2*: 22,503–24,275; ITS: 24,276–24,860; LSU: 24,861–28,215.

Maximum parsimony phylogenetic trees were generated for each partition using PAUP v4a168 (Swofford, 2003) with tree bisection and reconstruction (TBR) branch swapping as algorithm. Each parsimony analysis consisted of heuristic searches with 100 random taxon additions and were performed on parsimony-informative, unordered, and equally weighted characters. Gaps were treated as new character states and a maximum of 1,000 equally most parsimonious trees were allowed. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Other statistical measures calculated and recorded were tree length, consistency index, retention index and rescaled consistency index (TL, CI, RI and RC, respectively). The robustness of the obtained trees was evaluated by 1,000 bootstrap replications using the same heuristic searches as in the original analyses. Resulting trees were viewed in Geneious Prime v2022 (https://www.geneious.com, Kearse et al., 2012) and prepared for layout using Adobe Illustrator vCC 2023. The resulting trees can be found in Suppl. Fig. S1, with the exception of *tef1* phylogeny which is presented in Fig. 2. Statistics for the maximum parsimony phylogenetic analyses are available in Suppl. Table S3. The alignment and phylogenetic trees were deposited in figshare (https://doi.org/10.6084/m9.figshare.25187918).

2.3. Phylogenetic analysis based on BUSCO genes

The phylogenetic relatedness of 23 isolates was determined based on 2,996 conserved single copy orthologs identified using BUSCO v5.3.2 using the *Dothideomycetes* odb10 database (Manni et al., 2021). Protein sequences were aligned using MAFFT v7.453 (Katoh et al., 2002) and the maximum-likelihood phylogeny was determined using IQ-TREE v2 (Minh et al., 2020b) with 1,000 bootstraps and the setting rcluster 5 and the TESTMERGE modelfinder. Because bootstrap supports reach a maximum relatively easily with large datasets, we sought to provide a more robust support and therefore calculated gene concordance factors (gCF) and site concordance factors (sCF); site concordance was

calculated based on maximum likelihood (—-scfl) for 100 quartets. The phylogenetic tree was visualized using iTol (Letunic and Bork, 2021). The SplitsTree network was created for the BUSCO gene alignment using SplitsTree v4.17.0 (Huson and Bryant, 2006).

2.4. Whole-genome analyses

To calculate the amount of shared material between the strains, all-vs-all whole-genome alignments were made using PROmer v3.0 (Kurtz et al., 2004). For each pairwise alignment, bedtools coverage was used to calculate the coverage per window of 500 bp. Isolates were clustered based on pairwise identity using the hierarchical clustering and visualized using seaborn v0.11.2 (Waskom, 2021).

2.5. Mitochondrial data

Mitochondrial assemblies were generated and annotated using the JGI pipeline (Suppl. Table S2, (Haridas et al., 2018). We were able to generate complete and high-quality mitochondrial assemblies for 13 of the strains. To generate a phylogenetic tree based on the amino acid sequence alignments of mitochondrial genes, 13 genes were selected based on their presence in all genomes and the quality of the annotation data, these are: *atp6, atp9, cob, cox1, cox2, nad1, nad2, nad3, nad4, nad4L, nad5, nad6,* and *rps3*. The concatenated alignment consisted of the 13 *Phyllosticta* isolates and the outgroup taxon and the partitions were as follow: *atp6*: 1–263; *atp9*: 264–326; *cob*: 327–620; *cox1*: 621–1,199; *cox2*: 1,200–1,484; *nad1*: 1,485–1,863; *nad2*: 1,864–2,542; *nad3*: 2,543–2,659; *nad4*: 2,660–3,164; *nad4L*: 3,165–3,257; *nad5*: 3,258–3,967; *nad6*: 3,968–4,189; *rps3*: 4,190–4,703. The maximum-likelihood phylogeny was determined using IQ-TREE v2.1.3, with 1,000 bootstrap replicates and the default MFP modelfinder (Kalyaanamoorthy et al., 2017; Minh et al., 2020b; Nguyen et al., 2015). The phylogenetic tree was visualized using Geneious Prime v2022 (Kearse et al., 2012, https://www.geneious.com) and prepared for layout using Adobe Illustrator vCC 2023.

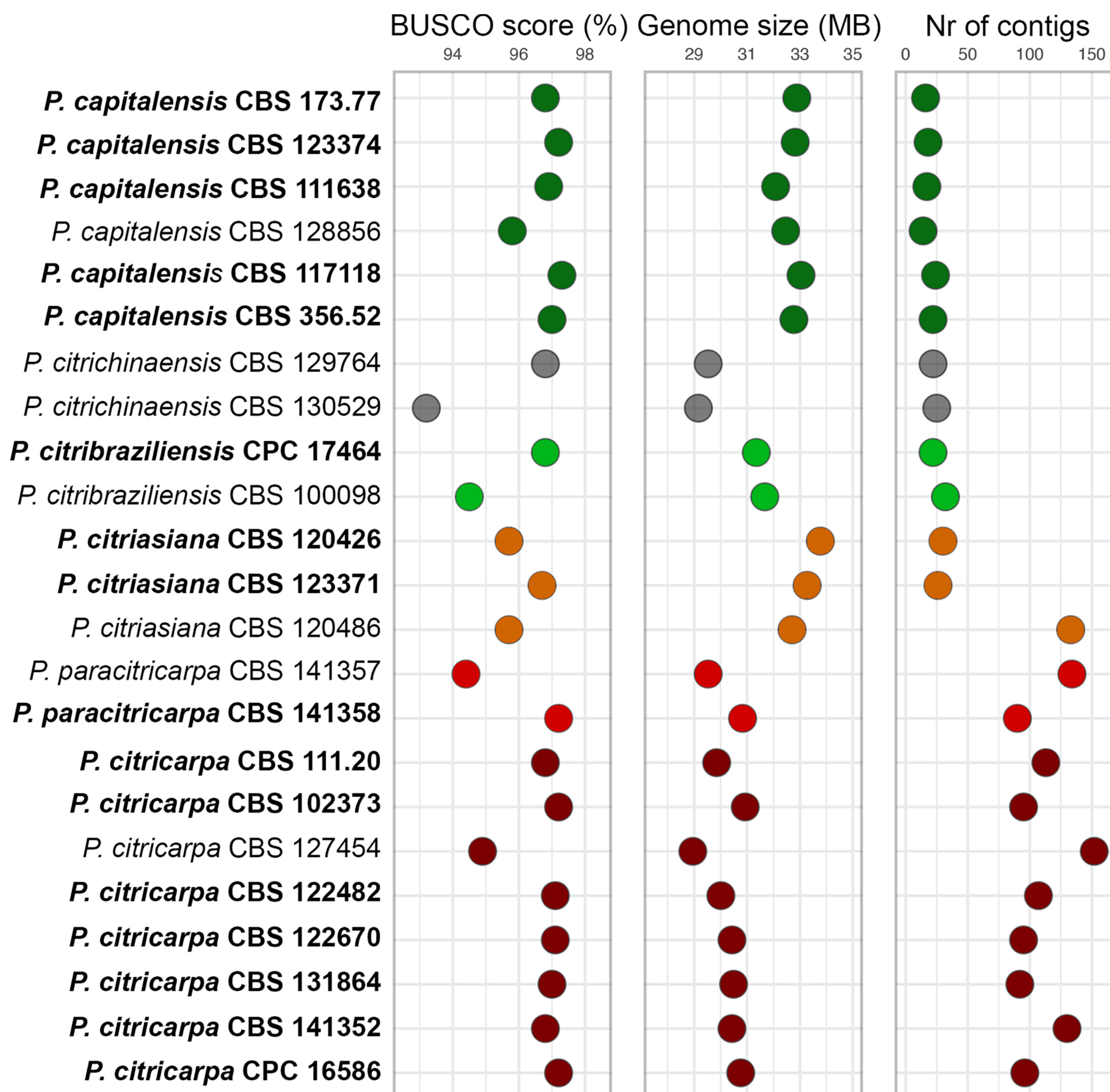

Fig. 1. Genome assembly quality statistics of 23 *Phyllosticta* genomes. The genome assemblies of 23 *Phyllosticta* strains were compared. The genomes of 16 strains that were newly sequenced and assembled are indicated in bold, and these data were supplemented with seven publicly available genome assemblies (see Suppl. Table S1 for an overview). For each sequenced *Phyllosticta* strain, the BUSCO score, genome size, and number of contigs are shown. Strains in green are considered to be endophytic, strains in red and orange are pathogenic, and strains in grey are ambiguous or intermediate (Buijs et al., 2022; Glienke et al., 2011; Wang et al., 2012; Wikee et al., 2013).

2.6. Mitochondrial genome alignment

Clinker v0.0.28 (Gilchrist and Chooi, 2021) was used to align the mitochondrial protein (amino acid) sequences of *Phyllosticta* species, using those alignments as ankers to align the mitochondrial genomes, and show the best match and the identity between genes.

3. Results

3.1. Pigment production is inconsistent across *P. paracitricarpa* strains and 16 newly sequenced genome assemblies are of good quality for comparative genomics

One of the characteristics proposed to distinguish *P. citricarpa* and *P. paracitricarpa* is the production of a yellow pigment on MEA by *P. paracitricarpa* strains (Guarnaccia et al., 2017). However, when we grew *P. citricarpa* and *P. paracitricarpa* for two weeks on MEA, some *P. paracitricarpa* strains did not produce a yellow pigment (Suppl. Fig. S2), which is not in line with earlier reports. These results demonstrate that this morphological feature is not a reliable basis to distinguish the two clades. To determine the relationship between *Citrus*-colonizing *Phyllosticta* species, and to be able to assess infraspecific variation, eight new genomes of *P. citricarpa* and *P. paracitricarpa* were sequenced, assembled, and compared to those of four related *Phyllosticta* species (*P. capitalensis, P. citriasiana*, *P. citribraziliensis*, and *P. citrichinaensis*), for which we also generated eight additional genome assemblies. The assembled genomes varied in size from 29 to 34 MB, and genome completeness assayed with BUSCO genes yielded BUSCO scores between 93 and 98 %, indicating adequate quality for comparative genomics (Fig. 1). The number of contigs varied between 14 and 152, with the genomes of *P. citricarpa* and *P. paracitricarpa* generally being much more fragmented compared to the other species' assemblies. With the

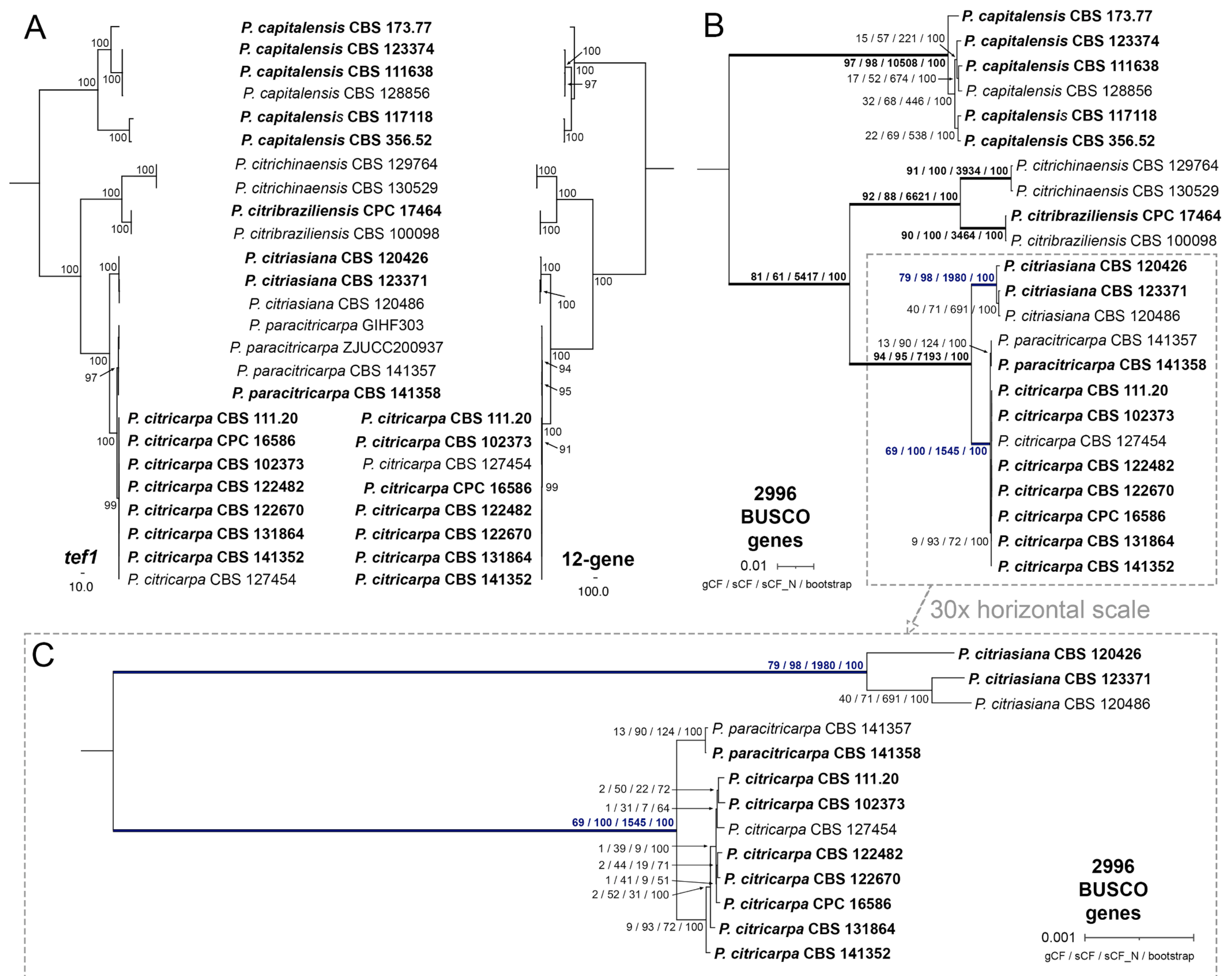

Fig. 2. Phylogenetic and phylogenomic analyses demonstrate that *Phyllosticta* infraspecific variation is larger than the interspecific variation between *P. citricarpa* and *P. paracitricarpa*. Newly sequenced genomes are shown in bold. **A.** Phylogenetic trees based on full-length barcoding genes. The left-hand tree is based on *tef1* only, the right-hand tree is based on a concatenated alignment of 12 barcoding gene regions: *tef1, actin, CaM, chs1, gapdh, his3*, ITS, LSU, *mcm7, rpb1, rpb2*, and *tub2*. For details on the analysis see Suppl. Table S3. The most parsimonious trees were generated using PAUP (Swofford, 2003). Only bootstraps above 80% are shown. The two trees are nearly identical with the exception of branch supports and some strains swapping positions within the *P. citricarpa* clade. **B.** Phylogenomic tree based on 2,996 BUSCO genes (1,905,363 aligned amino acids) from the class *Dothideomycetes*. Support values of clades are: gene concordance factor (gCF) / site concordance factor (sCF) / number of informative sites for sCF (sCF_N) / bootstrap. Branches with a gCF above 80% are shown in bold, branches with a gCF 60 – 80% are shown in bold and dark blue. **C.** Part of the tree from (B) but at 30x horizontal scale to allow visualization of leaves within the *P. (para)citricarpa* clade.

exception of *P. citriasiana* culture CBS 120486, which was sequenced using short-read sequencing technology (Illumina), all strains were sequenced using the same PacBio long-read sequencing platform at the Joint Genome Institute (JGI).

3.2. Phylogenetic and phylogenomic analysis show that interspecific variation between P. paracitricarpa and P. citricarpa is less than infraspecific variation in other Phyllosticta species

To assess to which extent the addition of the newly sequenced genomes challenges the established phylogeny of *Phyllosticta* species, we initially constructed a maximum parsimony phylogenetic tree based on 12 full-length gene regions. Of the genes used, *tef1* is most relevant, as it was previously used to distinguish *P. citricarpa* from *P. paracitricarpa* (Fig. 2A, left tree; Guarnaccia et al., 2017; Zajc et al., 2023). The other genes included were *actin, CaM, chs1, gapdh, his3,* ITS, LSU, *mcm7, rpb1, rpb2,* and *tub2*, which are all commonly used in phylogenetic analyses (Suppl. Fig. S1; Crous et al., 2021). To test whether including *P. paracitricarpa* strains from another geographic origin would affect the phylogeny, we also included two Chinese *P. paracitricarpa* strains that were made available online in 2023 (Suppl. Table S1, Guarnaccia et al. 2017, Ioos et al. 2023). Both the *tef1* as well as the 12-gene phylogenetic tree clearly separate at least five species: *P. capitalensis*, with a rather high level of infraspecific variation, *P. citrichinaensis*, *P. citribraziliensis*, and *P. citriasiana* with a lower level of infraspecific variation, and the *P. citricarpa/P. paracitricarpa* clade (Fig. 2A). The four *P. paracitricarpa* strains form a stable clade within the larger *P. citricarpa* clade, which is consistent with earlier phylogenetic studies (Guarnaccia et al., 2019, 2017). The level of variation between the *P. citricarpa* and *P. paracitricarpa* clades is similar to the variation within the *P. citriasiana* clade, and even smaller than the variation within the *P. capitalensis* clade. To quantify the amount of variation within a species, we determined the number of sequence changes from the most recent common ancestor (MRCA) to each strain within that species. For instance, the highest number of changes in a strain of *P. citriasiana* compared to the MRCA of *P. citriasiana* is 28 (Suppl Fig S3). Within *P. capitalensis,* this number is a ten-fold higher reaching up to 261 changes. The highest number of changes of any *P. citricarpa* strain as compared the MRCA of both *P. citricarpa* and *P. paracitricarpa* is 12, and for *P. paracitricarpa* it is 13. The variation between *P. citricarpa* and *P. paracitricarpa* is thus lower than the infraspecies variation within *P. citriasiana* or *P. capitalensis*.

To include more data in the phylogenetic analysis, and thereby provide a more robust basis, we made use of nearly 3,000 conserved BUSCO genes to construct a phylogenomic tree (Fig. 2B). In addition to bootstrap support values which reach the maximum of 100 % relatively easily with large datasets, we also generated gene concordance factors (gCF) and site concordance factors (sCF) (Minh et al., 2020a). The gCFs indicate the percentage of BUSCO genes that are in concordance with the position of each clade in the tree. The clades that contain the species *P. capitalensis*, *P. citrichinaensis* and *P. citribraziliensis* have gCFs of 97, 91 and 90 %, respectively, while the clade that contains the three supposed species *P. citricarpa*, *P. paracitricarpa* as well as *P. citriasiana* has a similar (high) gCF of 94 % (Fig. 2B). The clades of *P. citriasiana*, and *P. citricarpa/P. paracitricarpa* have lower gCFs of 79 and 69 %, respectively, which means there is lower support to separate these clades, suggesting that these species might be more closely related than the others (Fig. 2B, C). The clades of *P. citricarpa* and *P. paracitricarpa* separately have extremely low gCFs of 9 and 13 % respectively, which means that the gCFs do not support the separation of these strains into distinct clades, while gCFs do support the separation of all other species. The low gCFs of the *P. citricarpa* and *P. paracitricarpa* clades can be explained by a high similarity: sCFs are relatively high (93 and 90 %), but the sCF_Ns (number of informative sites) are only 124 and 72 (Fig. 2B, C). This is very low compared to the number of informative sites for other species clades such as 1,980 for *P. citriasiana*, 3,464 for *P. citribraziliensis*, and even 10,508 for *P. capitalensis*. In addition, the sCF_Ns within other species clades are higher, such as 691 within *P. citriasiana*, and up to 674 within *P. capitalensis*.

In addition, we assessed the presence of phylogenetic inconsistencies by performing a SplitsTree analysis (Suppl. Fig. S4, Huson and Bryant, 2006), which generates a split network to visualize inconsistencies in phylogenetic data caused by for instance hybridization or recombination events. This tree again confirmed the 12-gene tree topology; no inconsistencies are present and five clearly separated species can be observed (Suppl. Fig. S4), with *P. citricarpa* and *P. paracitricarpa* showing a level of variation that is comparable to infraspecies variation such as in *P. capitalensis* and *P. citriasiana*.

3.3. Genome-wide sharedness indicates variation within Phyllosticta citricarpa is larger than variation between P. citricarpa and P. paracitricarpa.

To include variation throughout the entire genome, including noncoding regions, in our analysis, we established the percentage of shared genetic material ('sharedness') between the different strains using all-vs-all whole-genome alignments. To do so, for each pairwise alignment the percentage of shared windows (500 bp) was calculated.

Whole-genome sequences within *Phyllosticta* species generally share between 81 and 95 % genetic material, with the least infraspecific shared genetic material between two strains observed within *P. capitalensis* (81 %) and *P. citriasiana* (83 %). In contrast, the highest sharedness between two strains was observed within *P. citricarpa* (95 %), *P. citribraziliensis* (94 %) and, interestingly, between *P. citricarpa* and *P. paracitricarpa* (94 %, Fig. 3A, Suppl. Table S4). Noticeably, whole-genome sharedness within *P. citricarpa* lies between 87 and 95 %, while sharedness between *P. citricarpa* and *P. paracitricarpa* lies between 88 and 94 %, indicating that the percentage of material shared between *P. paracitricarpa* and *P. citricarpa* lies entirely within the range of material shared within *P. citricarpa*. Moreover, the shared material of 94 % between *P. citricarpa* and *P. paracitricarpa* is higher than the highest observed shared genetic material within *P. paracitricarpa* (92 %), suggesting that one of the *P. paracitricarpa* strains is more similar to a *P. citricarpa* strain than to another *P. paracitricarpa* strain. In addition, hierarchical clustering based on the genome-wide shared genetic material clearly separated all species except for *P. citricarpa* and *P. paracitricarpa* strains that were intermixed within a single clade (Fig. 3C). To exclude the possibility that small, fragmented scaffolds and/or contigs that are only present in some genomes are distorting our analyses, we performed the same analysis but with the small scaffolds (<1,000 bp) removed. We did not observe any significant differences to the previous results (Suppl. Fig. S5).

We also performed a similar genome-wide analysis, but excluded unique windows, thereby only preserving informative windows in our analysis. On average, the percentage of genome-wide shared genetic material was much higher when compared to the previous analyses, with the least amount of infraspecies shared genetic material again observed within *P. capitalensis* (96.8 %) and *P. citriasiana* (98.3 %), and the highest sharedness within *P. citribraziliensis* (99.9 %, Fig. 3B, Suppl. Table S5). The percentage of shared genetic material within *P. citricarpa* and *P. paracitricarpa* was between 99.3 and 99.8 %, and 99.7 and 99.8 %, respectively, while the percentage of shared genetic material between *P. citricarpa* and *P. paracitricarpa* ranged from 99.1 to 99.5 %. In other words, the highest sharedness between a *P. citricarpa* and a *P. paracitricarpa* strain is 99.5 %, while the lowest sharedness within *P. citricarpa* is 99.3 %, and thus there are strains within the *P. citricarpa* clade that are more divergent from *P. citricarpa* than some *P. paracitricarpa* strains. In addition, using hierarchical clustering based on the sharedness, all strains are assigned to distinct clusters based on their species, while *P. citricarpa* and *P. paracitricarpa* were intermixed in a single clade (Fig. 3D), again demonstrating that *P. citricarpa* and *P. paracitricarpa* should be considered as one species.

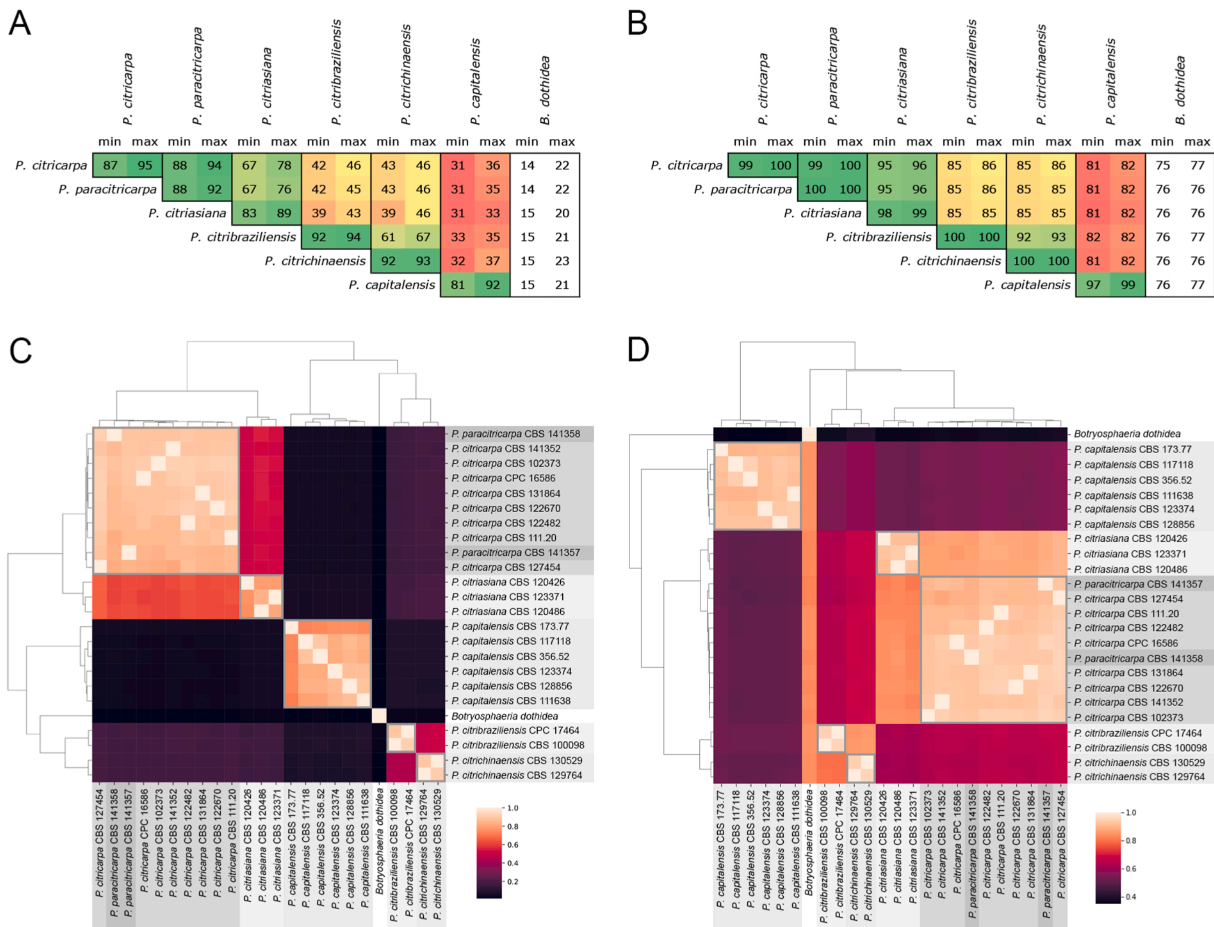

Fig. 3. Genome-wide sharedness indicates more variation within *Phyllosticta* species than between *P. citricarpa* and *P. paracitricarpa*. Minimum and maximum percentages of genetic material shared (i.e. 'sharedness') between different *Phyllosticta* species based on: **A.** whole genome sequences, and **B.** only the informative windows excluding unique windows. Heatmaps and hierarchical clustering based on sharedness of **C.** whole-genome sequences, and **D.** only the informative parts of the genomes.

3.4. All Phyllosticta species show large variations in their mitochondrial assemblies, but differences between P. citricarpa and P. paracitricarpa are minimal

Our analyses did not yet consider mitochondrial (mt)DNA, which is also used as a molecular marker in phylogenetic analyses since it is structurally well conserved (several genes will be found in practically any species), yet may have high mutation rates, meaning variation between species may be observed in mtDNA even if genomic DNA is highly similar (Kouvelis et al., 2008; Rubinoff and Holland, 2005; Sarma et al., 2017). We were able to generate high-quality single-contig mitochondrial genome assemblies using the JGI pipeline for several strains; strains for which we were not able to do so were excluded from the subsequent analyses.

Strikingly, we observed considerable differences in mitochondrial assembly size between different *Phyllosticta* species, with the largest assembly of 213 kb for *P. citriasiana* CBS 123371 being nearly twice as large as the smallest assembly of 118 kb for *P. capitalensis* CBS 173.77 (Fig. 4A, B). Difference in assembly length seems to correlate with lifestyles of the different species (Fig. 1), as species that are considered endophytic (*P. capitalensis*, *P. citribraziliensis*) have an average assembly length of 125.8 kb, whereas species that are considered pathogenic (*P. citriasiana*, *P. (para)citricarpa*) have an average assembly length of 209 kb (Fig. 4A). Notably, the difference in length is mainly caused by differences in gene length, as the total gene length of some species is also almost twice as long as those of other species (Fig. 4A). However, the total coding-sequence length across all species is very similar. It thus appears that this difference in length is almost entirely the result of differences in intron number and length (Fig. 4A), which can be clearly observed in *cob*, *nad1*, *nad5*, *cox3*, and especially for *cox1* (Fig. 4C). The difference in intron length may be explained by the presence of LAGLIDADG homing endonuclease genes (HEGs). Whereas endophytes contain on average 3.4 HEGs in the mitochondrial genome, pathogenic species contain on average 10.9 HEGs.

The level of conservation of mitochondrial sequences determines how well these sequences can be used to distinguish species. While most exons show a high level of conservation between different species, the introns may thus contain large insertions, sometimes up to several thousands of nucleotides long. Although these insertions vary between species, they seem to be conserved within species. In fact, mitochondrial assemblies within a species only show small variations such as SNPs or gaps up to a few nucleotides (mostly within *P. capitalensis*), and most species can easily be distinguished based on their mitochondrial intron content (Fig. 4B). However, unlike other *Phyllosticta* species, *P. citricarpa* and *P. paracitricarpa* were not easily distinguishable based on their mitochondrial assemblies as the sequences showed only minimal differences, and their intron structures were identical (Fig. 4B). We used the predicted mitochondrial annotations to generate a phylogenetic tree based on the amino acid alignment of 13 mitochondrial core genes (Fig. 4D). All species clearly clustered separately, with some variation present within *P. capitalensis*, but the *P. citricarpa* and *P. paracitricarpa* strains all clustered within one clade having completely identical amino acid sequences. Thus, the mitochondrial genome data corroborates the nuclear genome data, and suggest that *P. citricarpa* and *P. paracitricarpa*

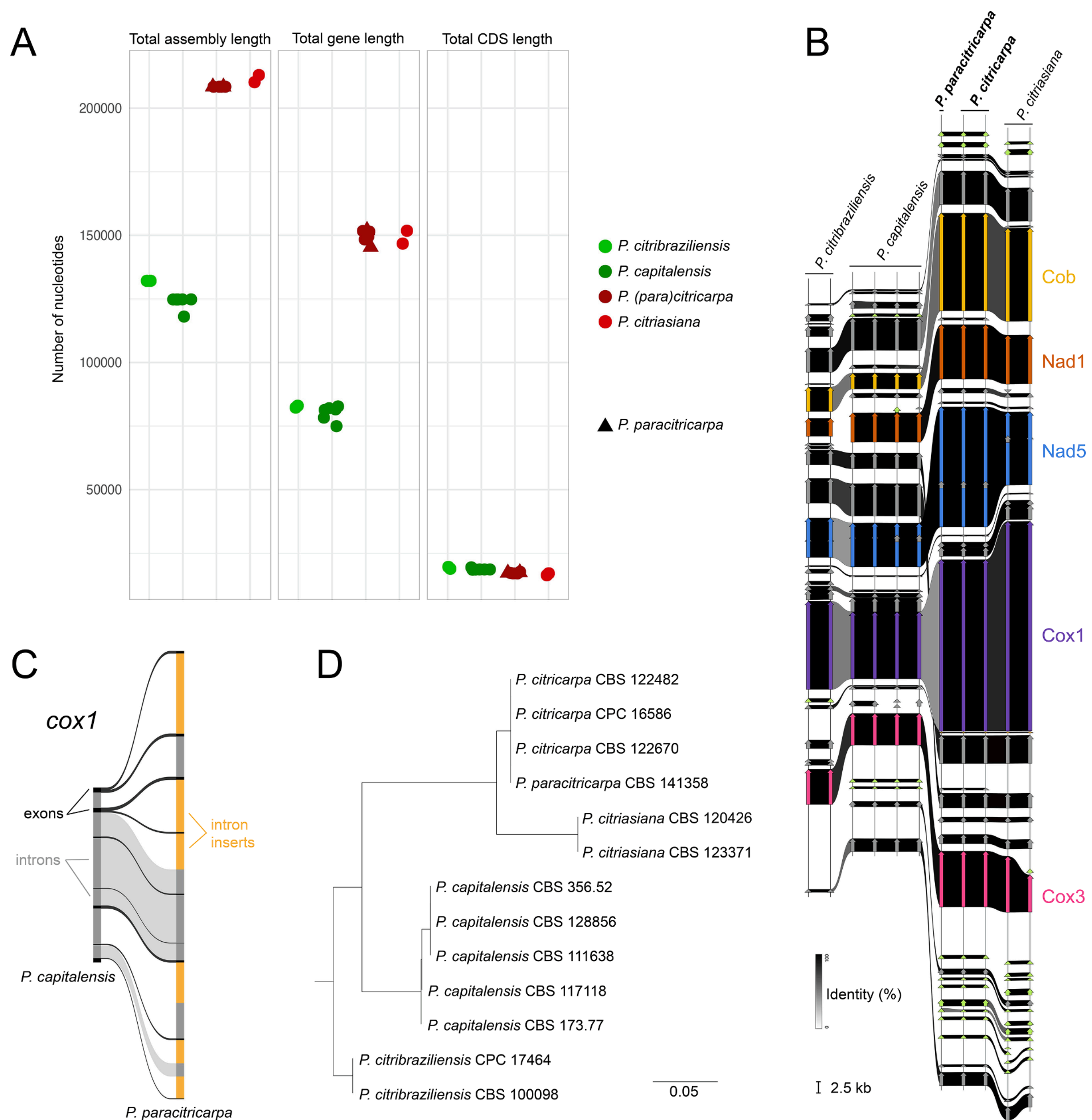

Fig. 4. ***P. citricarpa* and *P. paracitricarpa* cannot easily be distinguished based on mitochondrial assemblies, while other *Phyllosticta* species can. A.** Graph showing mitochondrial assembly statistics such as assembly length, gene length and coding sequence length. **B.** Clinker (Gilchrist and Chooi, 2021) alignment between mitochondrial assemblies of different *Phyllosticta* species. Connections indicate the best match between genes based on protein sequence identity (white 0% to black 100%). **C.** Schematic representation of an alignment between the *cox1* genes of *P. capitalensis* (left) and *P. paracitricarpa* (right). **D.** Phylogenetic tree obtained from the amino acid alignment of 13 mitochondrial genes.

should be considered as one species.

3.5. Taxonomy

Phyllosticta citricarpa (McAlpine) Aa, Stud. Mycol. 5: 40. 1973.

Basionym: *Phoma citricarpa* McAlpine, Fungus Diseases of Citrus trees in Australia: 21. 1899.

New synonym: *Phyllosticta paracitricarpa* Guarn. & Crous, Stud. Mycol. 87: 177. 2017.

Additional synonyms: See MycoBank (https://www.mycobank.org/).

Note: *Phyllosticta paracitricarpa* is reduced to synonymy with *P. citricarpa* based on the data presented here.

4. Discussion & conclusion

To be able to effectively manage plant diseases, it is of vital importance to properly distinguish dangerous plant pathogens from less threatening ones. This is particularly important for quarantine organisms, since fast and correct identification may prevent further spread. In the case of the quarantine fungus *P. citricarpa* and its close non-quarantine relative *P. paracitricarpa*, discrimination between the two clades is challenging, albeit not impossible (Ioos et al., 2023). Although these two clades were described as distinct species based on some morphological and a few nucleotide differences (Guarnaccia et al., 2017), their high degree of similarity raised the question as to whether these are indeed two distinct species. We therefore sought to determine how much variation is present in the genomes of the *Phyllosticta* species

that colonize citrus, and whether the interspecific variation between *P. citricarpa* and *P. paracitricarpa* falls within the limits of such infraspecific variation or not. Using several different genomic methods, such as phylogenomics using nearly 3,000 conserved single-copy ortholog genes, whole-genome comparisons, and a study of the mitochondrial assemblies, we determine that infraspecific variation within *Phyllosticta* species is larger than the variation between *P. citricarpa* and *P. paracitricarpa*. Based on these data, we therefore conclude that *P. citricarpa* and *P. paracitricarpa* should be considered as one species.

A phylogenetic analysis based on nearly 3,000 genes gave results highly similar to an analysis using only 12 gene regions, which demonstrates the sufficiency of these genes for phylogenetic and taxonomic purposes. The desired parts of the genome for such analyses are those that are somewhat conserved so that they can be found in different strains or species, but which are also variable enough to be able to distinguish species or strains from each other. Many conserved genes, including the commonly used barcoding genes, fit these qualifications. These are often belonging to the so-called housekeeping genes, i.e. genes involved in essential functions such as cell division or cell wall structure and thus by definition required for normal functioning of the cell. In cases where established barcoding genes may not work because of, for instance, conflicting signal or too little signal, supplementing with additional conserved genes may be necessary and useful (Balasundaram et al., 2015; Lücking et al., 2020; Pino-Bodas et al., 2013). However, our data shows that, when barcoding genes provide sufficient resolution and there are no conflicts, adding more genes may not necessarily be more informative, except for providing a stronger statistical branch support signal. Specifically, the use of gene concordance and site concordance factors (gCF/sCF) when using larger datasets provides a deeper insight into the potential presence of discordant signals that may be overseen when using a smaller set of genes. In conclusion, it is important to consider at least several (barcoding) genes and the resolution they provide before reaching conclusions on species delimitations or phylogenies.

Our data also showed the potential of mitochondrial assemblies for taxonomic purposes. Although *P. citricarpa* and *P. paracitricarpa* only showed minimal variations, other *Phyllosticta* species showed very large differences and could easily be distinguished. Rather than differences in gene sequence, differences in assembly length and gene structure, mainly intron presence and length, provided a valuable basis on which to distinguish species. Indeed, mitochondrial sequences are regularly used in taxonomic studies, most often as barcoding genes (Kouvelis et al., 2008; Kulik et al., 2020; Sarma et al., 2017). In the present study, we chose to perform the phylogenetic analyses on amino acid alignments as the high variability between species and absence/presence of introns made it impossible to obtain robust alignments for an analysis based on a nucleotide alignment of the mitochondrial genes. In addition, other studies have shown that fungal mitochondrial genomes can be highly dynamic in gene order and, as was shown here for *Phyllosticta*, may contain many and/or large introns (Paquin et al., 1997; Sandor et al., 2018). Our observation that HEGs contribute to mitochondrial genome size by increasing intron size is not unusual and has been described previously in other fungi (Fonseca et al., 2021; Megarioti and Kouvelis, 2020). However, whether the presence of extra HEGs in pathogenic *Phyllosticta* may have contributed to the emergence of a pathogenic lifestyle is unknown, and could be a subject for further study. Moreover, a detailed exploration of *Phyllosticta* mitochondrial assemblies could provide interesting insight into the development and evolution of the genus.

The presence of *P. citricarpa* in Europe remains a debated subject. Although Guarnaccia et al. (2017) collected multiple isolates from several citrus-growing areas in Europe, limited information is provided in respect to their methodology; however they do state that CBS symptoms were not observed. Later surveys conducted by the NPPOs of Italy, Greece, Malta and Portugal confirmed absence of CBS symptoms and the absence of *P. citricarpa* in leaf litter and asymptomatic fruit. Unfortunately, no detailed reports were published of these surveys, and thus it is difficult to conclude on how their methodologies might have differed from those of Guarnaccia et al. (2017). Remarkably, the guideline for surveys of *Phyllosticta citricarpa* as published by EFSA (Lázaro et al., 2020) does not include the sampling of leaf litter, even though all the European *P. citricarpa* strains reported by Guarnaccia et al. (2017) were isolated from leaf litter. This may be because the purpose of these surveys is to determine the presence of the disease Citrus Black Spot, which is a markedly different objective than determining the presence of the causal agent *P. citricarpa*, which can indeed be present asymptomatically in fruit, but may also survive and reproduce in leaf litter (Guarnaccia et al., 2019). Importantly, the presence of *P. citricarpa* may only lead to development of Citrus Black Spot symptoms under certain climatic conditions. The publication of the methodologies and results of surveys that include sampling of leaf litter could provide much clarity on the presence of *P. citricarpa* in Europe.

Although differences can be observed between *P. citricarpa* and *P. paracitricarpa*, these differences are smaller than the average *Phyllosticta* infraspecific variation, and thus these two species could be considered as one species. However, this only becomes apparent when considering multiple species of the genus, and multiple strains per species in an analysis, and thus it is very important to always place species in the context of a broader phylogenetic lineage by including more distant relatives in a genus or family. In our analysis, *P. capitalensis* showed the most infraspecific variation. In fact, based on our data, one could argue that *P. capitalensis* should not be considered as a single, but in fact several species. In other words: if we were to consider the degree of infraspecies variation of *P. capitalensis* as a 'threshold' for all *Phyllosticta* species, *P. citricarpa*, *P. paracitricarpa*, and *P. citriasiana* should be considered as a single species as well. However, *P. citriasiana* appears to have a particularly distinct host range and causes slightly different symptoms as compared to *P. citricarpa*, meaning there are notable biological distinctions between these two species. Indeed, including biological data in taxonomic studies is not uncommon (Chethana et al., 2021; Lücking et al., 2020). In addition, the interspecific variation between *P. citricarpa* and *P. citriasiana* is much larger than the infraspecific variation of both species, e.g. there is a barcoding gap present (Meyer and Paulay, 2005). It therefore appears unwise at present to consider them as a single species. If we were to apply a single 'threshold' of infraspecific variation that is allowed to exist in all species within *Phyllosticta,* and *P. citriasiana* should be considered a separate species, by the same threshold, *P. capitalensis* can then not be considered as one species. Several previous authors have in fact reported cryptic species in *P. capitalensis* in the past (see review by Norphanphoun et al., 2020), but even these are often difficult to distinguish using the standard multilocus phylogenies. However, a significant difference between *P. capitalensis* and the other *Phyllosticta* species is that *P. capitalensis* has a very broad host range of which it is frequently an ubiquitous, cosmopolitan endophyte and the larger variation within this species may therefore represent different host specializations within one species. Indeed, the strains used in this study span different continents and hosts, and the high variability within *P. capitalensis* may therefore indicate its broad range in geographic origin and host (Suppl. Table S1). Current data are insufficient to resolve whether *P. capitalensis* should be considered as one, or perhaps multiple species. Future studies involving phylogenomic analyses of accepted species in the *P. capitalensis* species complex are needed to identify better barcoding genes with a higher resolving power to identify which species are truly distinct and which might be synonyms.

It is important to realize that the question we pose here is not whether we are ultimately able to distinguish *P. citricarpa* and *P. paracitricarpa*, because if one really wants to, it should be possible to distinguish even the closest genetic relatives based on small species-specific genetic differences such as SNPs, as demonstrated by Ioos and colleagues (Ioos et al., 2023). A very good application of such lineage-specific differences is the work done on the different lineages of the oomycete pathogen *Phytophthora ramorum*, for which is it useful to

distinguish lineages originating from different parts of the world (Gagnon et al., 2014; Søndreli et al., 2023). However, with *P. (para) citricarpa* the question should first be whether it is meaningful to distinguish strains from each other at the species level. Species concepts for fungi are complicated, and over 30 different concepts have been proposed (Lücking et al., 2020). However, essentially there are three different types of properties of a species that can be considered to delimit it from another species; biological, morphological, and molecular differences.

Biological differences may for instance be the ability to mate or to colonize certain hosts. To use the (in)ability to mate in species delimitation for fungi can be challenging, as two lineages that cannot mate are not necessarily different species. For instance, mating may be very rare, could be dependent on environmental cues that may be unknown, and two distinct mating types may be necessary for successful reproduction (Li et al., 2020; Nagel et al., 2018; Yurkov et al., 2015). In addition, phylogenetically distinct species may hybridize (Samarasinghe et al., 2020). For *P. citricarpa* and *P. paracitricarpa*, biological differences do not provide a basis to delimit the species. Specifically, they can be found on the same host plants; *P. paracitricarpa* was isolated from *C. sinensis* and *C. limon* in China (Wang et al., 2023, 2012), while in this study we used *P. citricarpa* strains that were isolated from the same hosts from Argentina, Australia, Portugal, the USA and Zimbabwe. Although it is likely that they can mate due to their high similarity, this has not been demonstrated. However, it was shown that both species are heterothallic and that their mating-type loci are highly similar (Petters-Vandresen et al., 2020). The ability of *P. paracitricarpa* to cause disease symptoms similar to those caused by *P. citricarpa* remains uncertain. Wang et al. (2023) performed detached fruit assays using a single representative strain per species to prove Koch's postulates, and concluded that *P. paracitricarpa* strains are able to cause symptoms similar to those of *P. citricarpa*. However, it is unclear to which *tef1* clade (*citricarpa* or *paracitricarpa*) the used representative strain belongs. Guarnaccia et al. (2017) also used a detached fruit assay to show that artificially inoculated sweet orange fruits develop similar symptoms when inoculated with *P. citricarpa* and *P. paracitricarpa*. Whether disease symptoms of *P. paracitricarpa* exist in nature is unclear; Wang et al. (2012) lists numerous strains isolated from spots on *Citrus sinensis* fruits in China as "*P. citricarpa* subclade-II" (Wang et al., 2012), some of which could also be considered as *P. paracitricarpa* based on the *tef1* definition. This would imply that this species causes symptoms in nature. Considering the genetic similarity between *P. citricarpa* and *P. paracitricarpa* it seems logical that *P. paracitricarpa* would be able to cause similar symptoms, but to the authors' knowledge there are currently no data available to unequivocally support this claim. To be able to separate the two clades based on a difference in pathogenicity, more studies would have to be performed.

A second property that is often used in fungal species delimitation is morphology. However, fungal morphology is not always straightforward either as fungi may demonstrate several phenotypically distinct forms, often related to different parts of their life cycle (Bruckart et al., 2010; Wingfield et al., 2012). Morphological characteristics do not provide a criterium on which to distinguish *P. citricarpa* and *P. paracitricarpa* either, as their morphology is highly similar. Although average sizes may differ, such as those of conidiophores and conidia, sizes do overlap and may represent variation within a species. Culture characteristics are also frequently employed to help separate species. On MEA, fungal colonies of *P. paracitricarpa* appear yellow, becoming leaden-grey in the centre and yellow at the margin, while *P. citricarpa* colonies are olivaceous-grey (Guarnaccia et al., 2017). However, we show in this publication that not all *P. paracitricarpa* strains share this characteristic. Thus, these characteristics currently do not provide a basis to separate these clades either.

The third property on which to distinguish species is molecular characteristics. However, fungal genomes may be very diverse, with some species having much higher levels of infraspecific variation than others. Barcoding gaps, the absence of an overlap between levels of infra- and interspecific variation, may be present between species (Meyer and Paulay, 2005). We show in this publication that other *Phyllosticta* species have higher infraspecific variation than there is variation between *P. citricarpa* and *P. paracitricarpa*, indicating they should be considered one species. We included a variety of *P. citricarpa* strains from different geographical origins and hosts, thus variation of *P. citricarpa* is well represented in this study. In addition, we included the ex-type strain for *P. paracitricarpa* and included strains from different geographical origins in our 12-gene analyses, showing that addition of more diverse strains of *P. paracitricarpa* does not change the tree topology. In addition, results from both Ioos et al. (2023) and Wang et al. (2023) also suggest that an increased sampling does not dramatically increase the resolution between the two clades. On the contrary, the increased genetic variation being introduced with more strains results in less clade-specific polymorphisms and thus even more diffused clade boundaries. The genomic data shown here therefore provides a solid basis to combine *P. citricarpa* and *P. paracitricarpa* into one species, and does not provide any arguments to keep them separate at the species level. Thus, there is currently no basis – whether it be biological, morphological, or molecular – to distinguish them as separate species, and we should consider these strains to represent the same pathogen.

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6. Data statement

All genomes that were newly sequenced and assembled in this study are available at https://mycocosm.jgi.doe.gov/Phyllosticta. All supplementary data have been deposited at figshare (doi: 10.6084/m9.figshare.25187918).

CRediT authorship contribution statement

Valerie A. van Ingen-Buijs: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project Administration. **Anouk C. van Westerhoven:** Visualization, Resources, Methodology, Formal analysis, Data curation. **Petros Skiadas:** Visualization, Formal analysis, Data curation. **Xander C.L. Zuijdgeest:** Investigation. **Sajeet Haridas:** Formal analysis, Data curation. **Christopher Daum:** Data curation. **Kecia Duffy:** Data curation. **Jie Guo:** Data curation. **Hope Hundley:** Data curation. **Kurt LaButti:** Data curation. **Anna Lipzen:** Data curation. **Jasmyn Pangilinan:** Data curation. **Robert Riley:** Data curation. **Jie Wang:** Data curation. **Mi Yan:** Data curation. **Francis Martin:** Funding acquisition. **Kerrie Barry:** Data curation. **Igor V. Grigoriev:** Data curation. **Johannes Z. Groenewald:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Formal analysis, Data curation. **Pedro W. Crous:** Funding acquisition, Supervision, Writing – review & editing. **Michael F. Seidl:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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