Analysis of the Taxonomy and Pathogenic Factors of Pectobacterium aroidearum L6 Using Whole-Genome Sequencing and Comparative Genomics

Peidong Xu¹²†, Huanwei Wang†, Chunxiu Qin¹, Zengping Li¹, Chunhua Lin¹, Wenbo Liu¹* and Weiguo Miao¹*

¹Key Laboratory of Green Prevention and Control of Tropical Plant Diseases and Pests, Ministry of Education, College of Plant Protection, Hainan University, Haikou, China, ²School of Life Sciences, Hainan University, Haikou, China

Soft rot pectobacteria are devastating plant pathogens with a global distribution and a broad host range. Pectobacterium aroidearum L6, previously isolated from leaves of Syngonium podophyllum, is a pectolytic bacterial pathogen that causes typical soft rot on S. podophyllum. There is a shortage for genome data of P. aroidearum, which seriously hinders research on classification and pathogenesis of Pectobacterium. We present here the complete genome sequence of P. aroidearum L6. The L6 strain carries a single 4,995,896-bp chromosome with 53.10% G + C content and harbors 4,306 predicted protein-coding genes. We estimated in silico DNA–DNA hybridization and average nucleotide identity values in combination with the whole-genome-based phylogeny from 19 Pectobacterium strains including P. aroidearum L6. The results showed that L6 and PC1 formed a population distinct from other populations of the Pectobacterium genus. Phylogenetic analysis based on 16S rRNA and genome sequences showed a close evolutionary relationship among Pectobacterium species. Overall, evolutionary analysis showed that L6 was in the same branch with PC1. In comparison with 18 Pectobacterium spp. reference pathogens, strain L6 had 2,712 gene families, among which 1,632 gene families were identified as orthologous to those strains, as well as 1 putative unique gene family. We discovered 478 genes, 10.4% of the total of predicted genes, that were potentially related to pathogenesis using the Virulence Factors of Pathogenic Bacteria database. A total of 25 genes were related to toxins, 35 encoded plant cell-wall degrading enzymes, and 122 were involved in secretion systems. This study provides a foundation for a better understanding of the genomic structure of P. aroidearum and particularly offers information for the discovery of potential pathogenic factors and the development of more effective strategies against this pathogen.

Keywords: genome sequence, taxonomy, pathogenic gene, Pectobacterium aroidearum, comparative genomics
INTRODUCTION

Soft rot Pectobacteriaceae are considered to be one of the top ten most important agricultural phytopathogens (Mansfield et al., 2012). The family Pectobacteriaceae consisted of Pectobacterium spp. and Dickeya spp., formerly characterized as pectinolytic Erwinia spp. (Pérombelon, 2002; Charkowski et al., 2012; Adeolu et al., 2016). They cause destructive soft rot on a variety of field crops, fruits, ornamentals, and vegetables, including the staple food crop potato (Toth et al., 2003, 2011; Ma et al., 2007). Pectobacterium was first established in 1945 (Waldee, 1945; Ma et al., 2007). However, the classification of Pectobacterium spp. is not clear. Hauben et al. (1998) established the genus Pectobacterium that included three species and five subspecies: P. cacticidum, P. chrysanthemi, P. cypripedi, P. carotovorum subsp. atrosepticum, P. carotovorum subsp. betavasculorum, P. carotovorum subsp. carotovorum, P. carotovorum subsp. odoriferum, and P. carotovorum subsp. wasabiae. Subsequently, three subspecies of P. carotovorum were elevated into the species level, namely P. atrosepticum, P. odoriferum, and P. wasabiae (Gardan et al., 2003; Duarte et al., 2004; Nykyri et al., 2012). The classification of the genus Pectobacterium has been subjected to wide revision over the last decade, and it is likely that some of the genome-sequenced strains have been incorrectly assigned to P. carotovorum (Gardan et al., 2003; Khayi et al., 2016; Pritchard et al., 2016; Zhang et al., 2016). For instance, P. carotovorum subsp. carotovorum tends to serve as a catchall for pectobacteria isolates differing from the specific descriptions of the other pectobacteria taxa, and P. aroidearum was classified as a novel species in 2013 (Nabhan et al., 2013). The genome-sequenced strain PC1 (formerly classified as P. carotovorum subsp. carotovorum) is actually P. aroidearum under the new classification (Nabhan et al., 2013).

In the age of genomics, the Pectobacterium genus has been subjected to revision based on the development of bioinformatics. Several pectolytic bacterial strains were thought to belong to a novel Pectobacterium species after several taxonomic analyses including 16S rRNA gene sequence, DNA–DNA hybridization (DDH), genomics, and comparative genomics. These include P. actinidiae (Koh et al., 2012), P. polaris (Dees et al., 2017), P. peruviense (Waleron et al., 2018), P. zantedeschiae (Waleron et al., 2019), P. punjabense (Sarfraz et al., 2018, 2020), and P. aroidearum (Nabhan et al., 2013). The species of the genus Pectobacterium has 18 of child taxa with a validly published with correct name and four proposed species not yet validated based on The List of Prokaryotic names with Standing in Nomenclature1 (Table 1; Adeolu et al., 2016; Parte et al., 2020). Eleven species had complete genome data, and 7 had not complete assembly based on the National Center for Biotechnology Information (NCBI) genome database2 (Table 1). Only strain PC1 of P. aroidearum has its whole genome sequenced. Thus, there is a shortage of whole-genome data for P. aroidearum.

Currently, no methods and chemicals are effective in controlling Pectobacterium disease or preventing the spread of these pathogens (Zhang et al., 2017). In addition, planting patterns and storage conditions are not applicable for control of the disease (Yaganza et al., 2014). Bacterial strain L6, isolated from Syngonium podophyllum soft rot samples in Hainan Province, was recognized as P. aroidearum (Xu et al., 2020). There is a shortage for whole-genome data of P. aroidearum, which seriously hinders research on classification and pathogenesis of Pectobacterium. Genome

---

1https://www.bacterio.net/genus/pectobacterium
2https://www.ncbi.nlm.nih.gov/genome/?term=Pectobacterium
comparison revealed that most virulence genes are highly conserved in the *Pectobacterium* strains, especially for the key virulence determinants involved in the biosynthesis of extracellular enzymes and secretion systems (Li et al., 2019). The functional genomics methods are the effective ways to elucidate that this pathogen interacts with plants and causes disease (Toth et al., 2015). In this study, we sequenced the whole genome of *P. aroidearum* L6. Then, we compared it with genome analyses of 18 *Pectobacterium* reference strains. Furthermore, the genome annotation and comparative genomics analysis provided a foundation for a better understanding of the genomic structure of *P. aroidearum* and particularly offered information for the discovery of potential pathogenic factors and the development of more effective strategies against this pathogen.

**MATERIALS AND METHODS**

**Strain and Type Strain Genome Sequences**

Strain L6 was previously isolated from *S. podophyllum* soft rot samples in a plant nursery in the Haidian campus of Hainan University, Haikou, Hainan Province, China, in July 2019. Samples were collected from symptomatic *S. podophyllum* for bacterial isolation. Internal fragments containing symptomatic tissues were transferred to 1 mL of sterile distilled water, after 20 min, rinsed with 70% alcohol and sterile distilled water, and cultured onto Luria-Bertani (LB) medium for 48 h at 28°C to differentiate and characterize the bacterial pathogen. A total of 10 bacterial colonies were isolated from infected tissues. The isolated colonies were subcultured until the pure culture of the suspected bacterium was obtained. Two representative isolates (L5 and L6) were selected for further tests and one isolate preserved in Key Laboratory of Green Prevention and Control of Tropical Plant Diseases and Pests (Hainan University), Ministry of Education as *P. aroidearum* L6.

The pathogenicity of *P. aroidearum* L6 was previously reported (Xu et al., 2020). The putative pathogen was re-inoculated to confirm its pathogenicity in the incubator and field on the leaves of *S. podophyllum*. Typical symptoms of soft rot were observed 12–24 h after inoculation. *P. aroidearum* was re-isolated from a diseased leaf, fulfilling Koch's postulates. L6 was grown on LB broth for 12–24 h at 28°C. And genomic DNA of L6 was extracted by the Bacteria Genomic DNA Extraction Kit (Tiangen Biotech Co. Ltd., Beijing, China). Successful extraction of genomic DNA was confirmed by 0.8% agarose gels and quantified by Nanodrop ND-2000 (Thermo Fisher Scientific, United States). All complete genome sequences of *Pectobacterium* were retrieved from NCBI. Type strain genome sequences of 11 species which had complete assembly genome data were used, including *P. aroidearum* PC1 (GCF_000023605.1), *P. carotovorum* subsp. *carotovorum* JR1.1 (NZ_CP034237.1), *P. carotovorum* subsp. *carotovorum* 67 (NZ_CP034211.1), *P. atrosepticum* JG10-08 (NZ_CP007744.1), *P. atrosepticum* 21A (NZ_CP009125.1), *P. brassicaceae* SX309 (NZ_CP020350.1), *P. brassicaceae* 1,692 (NZ_CP047495.1), *P. odoriferum* JK2.1 (NZ_CP034938.1), *P. odoriferum* BC S7 (NZ_CP009678.1), *P. polaris* PZ1 (NZ_CP046377.1), *P. polaris* NIBIO1006 (NZ_CP017481.1), *P. actinidiae* KKH3 (NZ_JRMH01000001.1), *P. wasabiae* CFBP 3304 (NZ_CP015750.1), *P. parmentieri* HC (NZ_CP046376.1), *P. parmentieri* RNS 08–42-1A (NZ_CP015749.1), *P. versatile* 14A (NZ_CP034276.1), *P. versatile* 3–2 (NZ_CP024842.1), and *P. punjabense* SS95 (NZ_CP038498.1).

**Genome Sequencing and Assembly**

The *P. aroidearum* L6 genome was sequenced using a PacBio RS II platform and Illumina HiSeq 4,000 platform. For Illumina HiSeq sequencing, the fragments of 470 bp (with the approximate insert size of 350 bp) from adaptor-ligated DNA were recovered according to standard protocols. The libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument. Cutadapt (v1.9.1; Martin, 2011) was employed for quality control, and reads whose base groups have quality value below 20 at both ends, sequences containing more than 10% N base, or less than 75 bp in length were removed. The Illumina data were used for estimate and correction. Four SMRT cells zero-mode waveguide (a nano-optical device used to confine light to a small observation volume) arrays of sequencing were used in the PacBio platform to generate the subreads set. PacBio subreads (length < 1 kb) were removed. The program pbdagcon was used for self-correction. Draft genomic unitigs, which are uncontestated groups of fragments, were assembled using the Celera Assembler (Myers et al., 2000) against a high-quality corrected circular consensus sequence subreads set. To improve accuracy of the genome sequences, the Genome Analysis Toolkit (Mckenna et al., 2010) and SOAP tool packages (SOAP2, SOAPnsp, SOAPindel; Li et al., 2013) were used to make single-base corrections. To trace the presence of any plasmid, the filtered Illumina reads were mapped using SOAP to the bacterial plasmid database.

**Genome Component Prediction**

Gene prediction was performed on the L6 genome assembly using NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016; Haft et al., 2018; Li et al., 2021). The tRNA, rRNA, and sRNA recognition and the Rfam database (Gardner et al., 2009). Tandem repeats annotation was obtained using the Tandem Repeat Finder (Wirawan et al., 2010), and the minisatellite DNA and microsatellite DNA were selected based on the number and length of repeat units. The Genomic Island Suite of Tools GIST v1.0 was used for genomic island analysis with Island Path-DIOMB, SIGI-HMM, and Island Picker method (Hasan et al., 2012). Prophage regions were predicted using the PHAge Search Tool web server (Zhou et al., 2011) and CRISPR identification using CRISPR Finder (Grissa et al., 2007).

---

1. [https://github.com/PacificBiosciences/pbdagcon](https://github.com/PacificBiosciences/pbdagcon)
2. [https://www.broadinstitute.org/gatk/](https://www.broadinstitute.org/gatk/)
3. [http://www.ncbi.nlm.nih.gov/genomes/plasmid.html](http://www.ncbi.nlm.nih.gov/genomes/plasmid.html)
4. [http://tandem.bu.edu/trf/trf.html](http://tandem.bu.edu/trf/trf.html)
5. [http://phast.wishartlab.com/](http://phast.wishartlab.com/)
Phylogenetic Analysis
The maximum-likelihood (ML) phylogenetic analysis was inferred with FastME 2.1.6.1 including SPR postprocessing (Lefort et al., 2015) from the Genome BLAST Distance Phylogeny approach (GBDP) distances calculated using default settings from 16S rRNA gene sequences and genome sequences. Branch support was inferred from 1,000 pseudo-bootstrap replicates each. The trees were rooted at the midpoint (Farris, 1972) and visualized with PhyD3 (Kreft et al., 2017).

Average Nucleotide Identity and in silico DNA–DNA Hybridization Analysis
Pairwise comparison of their Average Nucleotide Identity (ANI) was based on BLAST+ (ANIB) from JSpeciesWS8 (Richter et al., 2015), while in silico DNA–DNA Hybridization (isDDH) was conducted using GBDP by the Type (Strain) Genome Server48 (Meier-Kolthoff and Göker, 2019). One-hundred distance replicates were calculated each. The DDH values and confidence intervals were calculated using the recommended settings of the Genome-to-Genome Distance Calculator (GGDC 2.1) for GGDC formula 2 (Meier-Kolthoff et al., 2013).

Comparative Genomics
Pectobacterium aroidearum L6 was compared with reference 18 Pectobacterium strains by using their genome sequence and gene sequence. BLAST core/pan genes of the six strains were clustered using CD-HIT (Fu et al., 2012) rapid clustering of similar proteins software with a threshold of 50% pairwise identity and 0.7 length difference cutoff in amino acids. Gene families were constructed using genes of L6 and reference strains. We carried out gene family TreeFam clustering treatment for the alignment results by Hcluster_sg software (Maqbool and Babri, 2007) and multiple sequence alignment with the clustered gene family using Muscle software (Edgar, 2004a,b). The phylogenetic tree was constructed using multiple sequence alignment results based on Muscle by the TreeBeST (Nandi et al., 2010) using the ML method.

Gene Annotation and Protein Classification
The function annotation is accomplished by analysis of protein sequences. We align genes with databases to obtain the highest quality corresponding annotations. Seven databases were used for general function annotation: Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa et al., 2016), Clusters of Orthologous Groups (COG; Galperin et al., 2015; Makarova et al., 2015), Non-Redundant Protein Database (NR), Swiss-Prot (UniProt Consortium, 2015), Gene Ontology (GO; Ashburner et al., 2000; Philip et al., 2014), TrEMBL (Apweiler et al., 2004), and Evolutionary Genealogy of Genes (EggNOG: Non-supervised Orthologous Groups; Huerta-Cepas et al., 2016) databases. Four databases were used for pathogenicity and drug resistance analysis. Virulence factors and resistance genes were identified based on the core dataset in Virulence Factors of Pathogenic Bacteria (VFDB; Chen et al., 2016) and the Antibiotic Resistance Genes Database (Liu and Pop, 2009); the other two databases were Pathogen Host Interactions (PHI; Winnenburg et al., 2006) and Carbohydrate-Active enZymes Database (CAZy; Levesqueur et al., 2013). Type III secretion system (T3SS) effector proteins were detected using EffectiveT3 (Vargas et al., 2012).

RESULTS
Genomic Features Among P. aroidearum L6 and Reference Strains
The P. aroidearum L6 genome was sequenced using a PacBio RS II platform and Illumina HiSeq 4,000 platform. The raw data were filtered, and this generated 1, 274 Mb of clean data. The reads were assembled into a genome and then assessed. We obtained a genome size of 4,995,896 bp, which consisted of a circular chromosome with no plasmid (Figure 1). GC-Depth analysis was performed on the assembly results to show the assembly length (4,995,896 bp), coverage (4,995,089), coverage (99.98%), reads usage percent (95.3%), and depth (260) of P. aroidearum L6. The average G + C content of the genome was 53.10%. A total of 4,306 putative protein-coding sequences, with total length of 4,298,622 bp (86.04% of total genome length; Table 2) and average length of 998.29 bp, were annotated on the P. aroidearum L6 genome. The genome encoded 77 tRNA operons and 40 sRNA genes. In addition, a total of 22 rRNA operons were present on the chromosome: eight 5S rRNAs, seven 16S rRNAs, and seven 23S rRNAs.

Phylogenetic Analysis of P. aroidearum L6
The 16S rRNA is the most useful and is commonly used as a molecular clock in the systematic classification of bacteria. Its evolution has good clock properties, being highly conserved in structure and function, and can well reflect the differences between different bacteria (Coenye and Vandamme, 2010). Thus, the accessibility of a large quantity of completely sequenced bacterial genomes allows the speedy and reliable determination of intragenomic sequence heterogeneity of 16S rRNA genes. The phylogenetic tree was inferred with FastME 2.1.6.1 from GBDP distances calculated from Pectobacterium species 16S rRNA and genome sequences. The branch lengths were scaled in terms of GBDP distance formula d5. The numbers on branches are GBDP pseudo-bootstrap support values >60% from 1,000 replications, with an average branch support of 91.1%. The delta value was 0.315 based on 16S rRNA and 0.156 based on genome sequences. A phylogram based on computing of the 16S rRNA showed a close relationship between both P. aroidearum L6 and PC1 genomes (Figure 2A). The phylogenetic relationships among P. aroidearum L6 and reference strains were determined based on genome sequence results (Figure 2B). The whole-genome-based phylogenetic tree showed that L6 was most closely related to P. aroidearum PC1.

---

1http://jspecies.ribohost.com/jspeciesws/
2http://tygs.dsmz.de/
Assessment of Taxonomy of Pectobacterium Species Using isDDH and ANI

We used isDDH and ANI to determine species delineation of Pectobacterium spp. Using empirical evidence based on classified species and their comparisons with DDH and ANI values, the same species were set at ≥70% identity in isDDH and ≥95% identity in ANI. The isDDH and ANI values (Figure 3) were consistent with their phylogenetic relationships (Figure 2), and members of the same phylogenetic clade also showed high isDDH and ANI values. The ranges of isDDH and ANI values for 18 Pectobacterium strains were 88–100 and 36–100. The P. aroidearum L6 and PC1 were evaluated to species level with isDDH = 83 and ANI = 98; furthermore, P. carotovorum subsp. carotovorum JR1.1 and 67 were not the same species with isDDH = 51 and ANI = 93, indicating that these two strains were misidentified.

Analysis of the Core Genome Among Pectobacterium Species and Reference Strains

The genomes of 18 Pectobacterium strains with P. aroidearum L6 were compared. The dispensable gene heatmap showed percentage of dispensable genes among strains. The identity matrix was
calculated based on BLASTP. The strains L6 and PC1 had the highest genetic similarity. Otherwise, *P. punjabense* SS95 also grouped together with *P. aroidearum* L6 and PC1 based on the dispensable gene heat map (Figure 4A). Analysis of pan genes among L6 strain and reference strains was carried out. There were 1944 genes shared by all of the bacteria. Among them, 132 genes were unique to L6 and 100 genes were unique to PC1 (Figure 4B). Research on special genes and core genes is important for the detection of functional differences and similarities between samples and provides molecular evidence for phenotype differences and similarities. A gene family is a group of genes that have the same ancestor and comprises more than two gene copies. The members of a gene family have similarity in structure and function, and the proteins produced are also similar. Gene families can be used to detect evolutionary history and gene differentiation. The gene family statistics showed that the final core genome was 1,632 gene families. One gene family was unique to L6, and one gene family was unique to PC1 (Figures 4C, D).

### Pathogenic Candidate Genes Obtained Through Gene Annotation Screening

We predicted genes associated with pathogenicity using GO, COG, KEGG, and especially VFDB, PHI, and T3SS, which are important databases for predicting bacterial pathogenicity. The VFDB database mainly focuses on the infectious agents among bacteria, mycoplasma, and chlamydia. A total of 478 (10.40%) genes were annotated to the VFDB definitions. The PHI database contains relationships between pathogens and hosts, and it predicted 432 (9.40%) genes. The T3SS has close relationship with Gram-negative pathogens and aids in determining infection mechanisms and toxicity at the molecular level. There were 723 (15.74%) predicted genes assigned to T3SS categories. Moreover, we screened the relevant genes...
FIGURE 2 | ML phylogenetic analysis for P. aroidearum L6 and reference strains. (A) Phylogenetic tree drawn using the 16S rRNA of Pectobacterium spp. (B) Phylogenetic tree drawn using the genomes of Pectobacterium spp. Bootstrap values are indicated in % of repetitions.
encoding plant cell-wall degrading enzymes (PCWDEs), toxins, and secretion systems for *P. aroidearum* L6 (Table 3). There were 25 genes related to toxins, and 35 genes encoded PCWDEs, including 28 encoding pectinases, three encoding cellulases, and four encoding proteinases. In addition, 122 genes were involved in six types of secretion systems: 14 genes in Type I (T1SS), 23 in Type II (T2SS), 29 in Type III (T3SS), 25 in Type IV (T4SS), none in Type V (T5SS), and 31 in Type VI (T6SS) secretion systems.

**DISCUSSION**

Plant bacterial soft rot is one of the destructive diseases of cabbage, tomato, and potato (Meng et al., 2017; Wang et al., 2017; Cui et al., 2019). And it always can cause more serious losses than any other bacterial disease (Qi et al., 2021). Most of the soft rot disease of vegetables is caused by *Pectobacterium* spp., one of the top ten bacterial plant pathogens. *Pectobacterium* spp. has attracted more attention about its wide distribution and diversity (He et al., 2021). *Pectobacterium* usually exists in soils with a broad range of hosts; thus, *Pectobacterium* species cause soft rot disease in plants of at least 16 dicotyledonous and 11 monocotyledonous angiosperm families (Ma et al., 2007; Nabhan et al., 2013). *Pectobacterium aroidearum* was classified as a novel species in 2013 (Nabhan et al., 2013). In previous studies, bacterial soft rot disease was caused by *P. aroidearum* in calla (*Zantedeschia aethiopica*; Nabhan et al., 2013), potato (*Solanum tuberosum*; Moretti et al., 2016), Chinese cabbage (*Brassica rapa*; Xie et al., 2017), zucchini (*Cucurbita pepo*; Moraes et al., 2017), konjac (*Amorphophallus konjac*; Sun et al., 2019), pepper (*Capsicum annuum*; Moraes et al., 2020), and carrot (*Daucus carota*; Tang et al., 2021). In our previous research, *P. aroidearum* as a pathogen on *S. podophyllum* was found in China (Xu et al., 2020). It is important to prevent the spread of this pathogen because many ornamental and edible plant species are susceptible to *P. aroidearum*.

The classification of the genus *Pectobacterium* has been subject to wide revision over the last decade. *Pectobacterium* spp. are highly phenotypically, genetically, and pathogenically
particularly heterogeneous, indicating a need for re-evaluation and a better classification of these species (Dees et al., 2017). Three subspecies of *P. carotovorum* were reclassified as one subspecies (*P. carotovorum* subsp. *carotovorum*), and *P. carotovorum* subsp. *odoriferum* and *P. carotovorum* subsp. *brasiliense* were reclassified as *P. odoriferum* and *P. brasiliense*, respectively, based on genomics (Liu and Filiatrault, 2020; Liu et al., 2020). A lot of genomes of *Pectobacterium* have been sequenced, annotated, and analyzed previously (Oulghazi et al., 2020; Pedersen et al., 2020; Jonkheer et al., 2021). But there was no information about the whole genome of *P. aroidearum*-type strain SCRI 109. At present, only strain PC1 of *P. aroidearum* has sequenced its whole genome (PC1 formerly classified as *P. carotovorum* subsp. *carotovorum*, the classification has not been corrected in the NCBI database; He et al., 2021). To understand the molecular mechanisms of taxonomy and pathogenic factors in *P. aroidearum*, the whole genome of L6 was successively sequenced in this study.
And it will be the first public report on the genome of *P. aroidearum*.

Comparison of genomes is an efficient method for classification and detection of bacterial and fungal pathogens (Pritchard et al., 2012; Bühlmann et al., 2013; Malapi-Wight et al., 2016; Tang et al., 2017; Van Dam et al., 2018). Zoledowska et al. (2018) researched the comparative genomic of 15 *P. parmentieri* strains and found the high genomic variation among *P. parmentieri* strains. Pédron et al. (2019) established the taxonomic status of six *Pectobacterium* strains based on phylogenetic data, ANI values, and isDDH results by comparative genomics and identified a novel species of the genus *Pectobacterium* named *Pectobacterium aquaticum*. He et al. (2021) analyzed comparative genomics of four *Pectobacterium* strains and obtained three kinds of highly conserved key pathogenic genes related to cell-wall degrading enzymes in *Pectobacterium* strain PC1, including 19 pectinase genes, 25 cellulase genes, and 22 protease genes. Zhang et al. (2016) compared 85 genomes of the genera *Dickeya* and *Pectobacterium* and found that at least ten tested genomes from these genera were misnamed in GenBank based on ANI, isDDH, and whole genome. In our study, L6 and PC1 were grouped in one population distinct from other populations of the *Pectobacterium* genus and we also found some strains were misnamed in GenBank. (*P. carotovorum* subsp. *carotovorum* JR1.1 and 67 were not the same species.) It is effective for re-evaluating current prokaryotic species definition and establishing a unified prokaryotic species definition frame by using whole-genome sequences for taxonomically challenging genera (Zhang et al., 2016; Qi et al., 2021).

Currently, once plants are infected, there is no effective method to control bacterial soft rot (Sun et al., 2019). It is also very possible to develop new control methods. By screening pathogenic genes based on whole-genome sequences of *Pectobacterium* species and analyzing the pathogenic mechanism at the molecular level, Zhang et al. (2017) discovered a total of 168 genes related to pathogenesis including nine specific genes encoding toxins on the genome of *P. atroseptica* JG10-08. Huang et al. (2019) selected five putative effectors from the genome of *P. carotovorum* subsp. *brasiliense* BZA12 and discovered that candidate effector A12GL002483 was localized in the cell nucleus and induced cell death. We discovered 478 genes, 10.4% of total predicted genes, that were potentially related to pathogenesis according to the VFDB database. Previous research has shown that soft rot pathogenesis basically relies on toxins, PCWDEs, and the secretion system. Toxins play a key role in the pathogenicity of *Pectobacterium* species. We discovered 29 genes related to toxins in *P. aroidearum* L6. Moreover, PCWDEs are crucial in three distinct pathogenic functions: degradation, nutrition, and feedback regulation (Franza et al., 2002; Yang et al., 2007). The pathogens benefit from the nutrients produced after degradation; these degradation products accumulate in the host and can induce bacteria to generate more enzymes (Zhang et al., 2017). Therefore, the production of PCWDEs is characteristic of infection by *Pectobacterium* species. The PCWDEs consist of pectinases, cellulases, and proteases. In this study, we identified a total...
of 36 genes encoding PCWDEs: 29 encoding pectinases, three encoding cellulases, and four encoding proteinases.

In addition to toxins and PCWDEs, secretion systems play a critical role in plant bacterial disease development. There are six types of secretion systems to export extracellular enzymes and effector proteins in bacteria (Lory, 1998). Through secretion systems, effectors can be transported inside the plant cell and promote rapid infection of the host plant (Holst et al., 1996). Among them, the Hrp gene cluster encoding the T3SS is particularly important in many Gram-negative pathogens; this is a multi-protein complex bacterial structure to deliver virulence effector proteins directly into plant cells (Tampakaki et al., 2004; Tam et al., 2007; Xie et al., 2019). In our study, there were 723 predicted genes assigned to T3SS categories. Furthermore, 122 genes were involved in the six types of secretion system in P. aroidearum L6 based on GO, COG, and KEGG: 14 in T1SS, 23 in T2SS, 29 in T3SS, 25 in T4SS, and 31 in T6SS. Pasanen et al. (2020) identified a novel species Pectobacterium parvum and found it contained SPI-1-type III secretion island by comparing between the genomes of Pectobacterium species. In the genome of Pseudomonas syringae, Kang et al. (2014) researched the role of T3SS effectors in the disruption of actin cytoskeleton and inhibition of endocytosis. In the genome of Shewanella sp., Alex and Antunes (2019) detected the genes encoding for T3SS core components and four copies of homologs of T3SS effector. Currently, the effectors of P. aroidearum pathogenesis have not been studied. All these genes in P. aroidearum L6 have potential virulence functions. Thus, further research on the pathogenic factors in L6 may reveal the mechanism of Pectobacterium species infection of plants.

CONCLUSION

The classification of the genus Pectobacterium has long been unclear. Pectobacterium spp. are highly phenotypically, genetically, and pathogenically particularly heterogeneous, can cause severe soft rot in plant hosts, and have a wide host range. Our results suggest that P. aroidearum L6 synthesizes and transports virulence factors. Moreover, 182 genes were involved in toxins, PCWDEs, and the secretion system. The results of this research will serve as a foundation for a better understanding of the genomic structure of P. aroidearum. The discovery of potential pathogenic factors can help in preventing spread and outbreak of this pathogen and providing effective biological measures against it.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request. The genome sequence of P. aroidearum L6, including all assemblies and annotations, generated for this study is available at NCBI GenBank with accession number CP065044. The 16S ribosomal RNA sequence is available with accession code MT120309.

AUTHOR CONTRIBUTIONS

WL and PX designed the experiments and wrote the manuscript. PX, HW, CQ, and ZL performed collection and bioinformatics analysis. CL and WM revised the manuscript. All authors read and approved the final manuscript.

FUNDING

This study was financially supported by the Hainan Major Research Fund of Science and Technology (grant no. ZDKJ201817), the National Natural Science Foundation of China (grant nos. 31560495, 31760499, and 31660033), the National Natural Rubber Industry Technical System (grant no. CARS-34-GW8), the Opening Project Fund of Key Laboratory of Biology and Genetic Resources of Rubber Tree, Ministry of Agriculture and Rural Affairs, P. R. China (grant no. RRI-KLOF201903), and the Hainan Province Graduate Student Innovation Research Project (grant nos. Hyb2018-07 and Hyb2019-02).
REFERENCES

Adelou, M., Alnajar, S., Naushad, S., and Gupta, R. S. (2016). Genome-based phylogeny and taxonomy of the ‘Enterobacteriales’: proposal for Enterobacteriales ord. nov. divided into the families Enterobacteriaceae, Erwiniaeaeaeam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morganellaceae fam. nov., and Budviciaceae fam. nov. Int. J. Syst. Evol. Microbiol. 66, 5575–5599. doi: 10.1099/ijsem.0.011485

Alex, A., and Antunes, A. (2019). Whole-genome comparisons among the genus Shevalinella reveal the enrichment of genes encoding ankyrin-repeats containing proteins in sponge-associated bacteria. Front. Microbiol. 10.5. doi: 10.3389/fmicb.2019.000055

Apweiler, R., Bairoch, A., Wu, C. H., Barker, W. C., Boeckmann, B., Ferro, S., et al. (2004). UniProt: the universal protein knowledgebase. Nucleic Acids Res. 32, D115–D119. doi: 10.1093/nkg/k3h131

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene ontology: tool for the unification of biology. Nat. Genet. 25, 25–29. doi: 10.1038/75556

Brenner, D. J., Steigerwalt, A. G., Miklos, V. G., and Fanning, G. R. (1973). Deoxyribonucleic acid relatedness among Erwinia and other Enterobacteriaceae: the soft-rot organisms (genus Pectobacterium Wäldke). Int. J. Syst. Bacteriol. 23, 205–216. doi: 10.1099/00207713-23-3-205

Bühlmann, A., Pothier, J. F., Tomlinson, J. A., Frey, J. E., Boonham, N., Smits, T. H. M., et al. (2013). Genomics-informed design of loop-mediated isothermal amplification for detection of phytopathogenic Xanthomonas arboricola pv. pruni at the intraspecific level. Plant Pathol. 62, 475–484. doi: 10.1111/j.1365-3059.2012.02654.x

Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., et al. (2012). The role of secretion systems and small molecules in soft-rot Enterobacteriaceae pathogenicity. Annu. Rev. Physiol. 50, 425–449. doi: 10.1146/annurev-phys-012810-140832

Chen, L. H., Zheng, D. D., Liu, B., Yang, J., and Jin, Q. (2016). VFDB 2016: hierarchical and refined dataset for big data analysis-10 years on. Nucleic Acids Res. 44, D964–D967. doi: 10.1093/nkg/dxv1239

Coenye, T., and Vandamme, P. (2010). Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. FEMS Microbiol. Lett. 228, 45–49. doi: 10.1111/j.1365-3059.2010.06711-7

Cui, W., He, P., Munir, S., He, P., He, Y., Li, X., et al. (2019). Biocontrol of soft rot of chinese cabbage using an endophytic bacterial strain. Front. Microbiol. 10.1471. doi: 10.3389/fmicb.2019.01471

Dees, M. W., Lyse, E., Rossman, S., Pernimov, J., and Burbreg, M. B. (2017). Pectobacterium polaris sp. nov., isolated from potato (Solanum tuberosum). Int. J. Syst. Evol. Microbiol. 67, 5222–5229. doi: 10.1099/ijsem.0.02348

Durante, V., de Boer, S. H., Ward, L. I., and de Oliveira, A. M. R. (2004). Characterization of atypical Erwinia carotovora strains causing blackleg of potato in Brazil. J. Appl. Microbiol. 96, 535–545. doi: 10.1111/j.1365-2672.2004.01713.x

Edgar, R. C. (2004a). MUSCLE: multiple sequence alignment with high accuracy and fast throughput. Nucleic Acids Res. 32, 1792–1797. doi: 10.1093/nkg/k3h340

Edgar, R. C. (2004b). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinf. 5:113. doi: 10.1186/1471-2105-5-113

Farris, J. S. (1972). Estimating phylogenetic trees from distance matrices. Am. Nat. 106, 453–668. doi: 10.2307/2459725

Franza, T., Michaud-Soret, L., Piquero, P., and Expert, D. (2002). Coupling of iron assimilation and peroxidin in Erwina chrysanthemi 3937. Mol. Plant-Microbe Interact. 15, 1181–1191. doi: 10.1094/MPMI.2002.15.11.1181

Fu, L., Niu, B., Zhu, Z., Zhu, Z. W., Wu, S., and Li, W. Z. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28, 3150–3152. doi: 10.1093/bioinformatics/bts565

Galperin, M. Y., Makarova, K. S., Wolf, Y. I., and Eugene, V. K. (2015). Expanded genome databases: a true mirror of biodiversity. Nucleic Acids Res. 43, D261–D269. doi: 10.1093/nkg/kzj223

Gardan, L., Gouy, C., Christen, R., and Samson, R. (2003). Elevation of three subspecies of Pectobacterium carotovorum to species level: Pectobacterium atrofaciens sp. nov., Pectobacterium betavasculorum sp. nov. and Pectobacterium vasaubae sp. nov. Int. J. Syst. Evol. Microbiol. 53, 381–391. doi: 10.1099/jjs.0.02423-0

Gardner, P. P., Dubh, J., Tate, J. G., Nawrocki, E. P., Kolbe, D. L., Lindgreen, S., et al. (2009). RFam: updates to the RNA families database. Nucleic Acids Res. 37, D136–D140. doi: 10.1093/nkg/k766
Comparative genomic analysis of Pectobacterium carotovorum subsp. brasiensiensis SX309 provides novel insights into its genetic and phenotypic features. BMC Genomics 20:486. doi: 10.1186/s12866-011-0431-0

Liu, Y. Y., and Filatratu, M. J. (2020). Complete genome sequence of the necrotrophic plant-pathogenic bacterium Pectobacterium brassicae 1692. Microbiol. Resour. Announc. 9:e00037-20. doi: 10.1128/MRA.00037-20

Liu, Y. Y., Helmann, T., Stodgill, P., and Filatratu, M. (2020). Complete genome sequence resource for the necrotrophic plant-pathogenic bacterium Pectobacterium carotovorum WPP14. Plant Dis. 105, 196–198. doi: 10.1094/PDIS-05-20-1059-A

Liu, B., and Pop, M. (2009). ARDB-antibiotic resistance genes database. Nucleic Acids Res. 37, D443–D447. doi: 10.1093/nar/gkn656

Lory, S. (1998). Secretion of proteins and assembly of bacterial surface organelles: shared pathways of extracellular protein targeting. Curr. Opin. Microbiol. 1, 27–35. doi: 10.1016/1369-5274(97)00139-2

Lowe, T. M., and Eddy, S. R. (1997). TRNAScan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 25, 955–964. doi: 10.1093/nar/25.5.955

Ma, B., Hibbon, M. E., Kim, H. S., Reedy, R. M., Yedin, I., and Breuer, J. (2007). Host range and molecular phylogenies of the soft rot Enterobacterial genera Pectobacterium and Dickeya. Phytopathology 97, 1150–1163. doi: 10.1094/PHYTO-97-9-1150

Makarova, K., Wolf, Y., and Koonin, E. (2015). Archaeal clusters of orthologous genes (archCOGs): an update and application for analysis of shared features between Thermotogales, Methanobacterales, and Methanobacteriales. N. Microbiol. 5, 818–840. doi: 10.3390/ lif5010018

Malapiti-Wright, M., Demers, J. E., Veltri, D., Marra, R. E., and Crouch, J. A. (2016). LAMP detection assays for boxwood blight pathogens; a comparative genomics approach. Sci. Rep. 6:26140. doi: 10.1038/srep26140

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sariyar, M., Ronal, M., et al. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. Mol. Plant Pathol. 13, 614–629. doi: 10.1111/j.1364-3633.2012.00804.x

Maqbool, O., and Babri, H. (2007). Hierarchical clustering for software architecture recovery. IEEE Trans. Softw. Eng. 33, 759–780. doi: 10.1109/TSE.2007.70732

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EBNet J. 17, 10–12. doi: 10.18406/ej.17.1.20

Mckenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., et al. (2010). The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303. doi: 10.1101/gr.107524.110

Meier-Kolthoff, J. P., Auch, A. F., Klenk, H. P., and Göker, M. (2013). Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinf. 14:660. doi: 10.1186/1412-480X-14-660

Meier-Kolthoff, J. P., and Göker, M. (2019). TGVS is an automated high-throughput platform for state-of-the-art genome-barcoding taxonomy. Nat. Commun. 10:2182. doi: 10.1038/s41467-019-10210-3

Meng, X. L., Chai, A., Shi, Y. X., Xie, X. W., Ma, Z. H., and Li, B. J. (2017). Emergence of bacterial soft rot in cucumber caused by Pectobacterium carotovorum subsp. brasiiliense in China. Plant Dis. 101, 279–287. doi: 10.1094/PDIS-05-16-0763-RE

Moraes, A. J. G., Baia, A. D. B., Souza, E. B., Peixoto, A. R., and Gama, M. A. S. (2020). First report of Pectobacterium aroidearum causing soft rot of pepper (Capsicum annuum) fruits in Brazil. Plant Dis. 104:3054. doi: 10.1094/PDIS-02-20-0403-PDN

Moraes, A. J. G., Souza, E. B., Mariano, R. L. R., Silva, A. M. F., Lima, N. B., Peixoto, A. R., et al. (2017). First report of Pectobacterium aroidearum and Pectobacterium carotovorum subsp. brasiiliensis causing soft rot of Cucurbita pepo in Brazil. Plant Dis. 101, 379–380. doi: 10.1094/PDIS-08-16-1168-PDN

Moretti, C., Fakhri, R., Cortese, C., De, V. P., Cerri, M., Geagela, L., et al. (2016). Pectobacterium aroidearum and Pectobacterium carotovorum subsp. carotovorum causal agents of potato soft rot in Lebanon. Eur. J. Plant Pathol. 144, 205–211. doi: 10.1007/s10658-015-0743-3

Myers, E. W, Sutton, G. G., Delcher, A. L., Dew, I. M., Fasulo, D. P., Flanigan, M. J., et al. (2000). A whole-genome assembly of Drosophila. Science 287, 2190–2194. doi: 10.1126/science.287.5461.2190

Nabhan, S., De Boer, S. H., Maes, E., and Wydra, K. (2013). Pectobacterium aroidearum sp. nov. a soft rot pathogen with preference for monocotyledonous plants. Int. J. Syst. Evol. Microbiol. 63, 2520–2525. doi: 10.1099/ijsem.0.046011-0
Xu, V. C., Serruto, D., Dziejman, M., Brierer, W., and Mekalanos, J. J. (2007). A type III secretion system in Vibrio cholerae translocates a formin/spire hybrid-like actin nucleator to promote intestinal colonization. Cell Host Microbe 1, 95–107. doi: 10.1016/j.chom.2007.05.005

Tampakaki, A. P., Fadoulouglou, V. E., Gazi, A. D., Panopoulos, N. J., and Kokkinidis, M. (2004). Conserved features of type III secretion. Cell. Microbiol. 6, 805–816. doi: 10.1111/j.1462-5822.2004.0043x

Tang, W. Q., Chang, C. Y., Lee, Y. J., and Chu, C. C. (2021). First report of Pectobacterium aroidearum causing bacterial soft rot of carrot in Taiwan. Plant Dis. 105:695. doi: 10.1094/PDIS-08-20-1824-PDN

Tang, J. T., Zheng, L., Jia, Q., Liu, H., Hsiang, T., and Huang, J. B. (2017). PCR markers derived from comparative genomics for detection and identification of the rice pathogen Ustilaginoidea virens in plant tissues. Plant Dis. 101, 1515–1521. doi: 10.1094/PDIS-08-16-1088-RE

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Navrocki, E. P., Zaslavsky, L., et al. (2016). NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res. 44, 6614–6624. doi: 10.1093/nar/gkw569

Toth, I. K., Bell, K. S., Holeva, M. C., and Birch, P. R. (2003). Soft rot erwiniae: from genes to genomes. Mol. Plant Pathol. 4, 17–30. doi: 10.1046/j.1364-3703.2003.00149.x

Toth, I., Humphris, S., Campbell, E., and Pritchard, L. (2015). Why genomics research on Pectobacterium and Dickeya makes a difference. Ann. J. Potato Res. 92, 218–222. doi: 10.1007/s12230-015-9446-8

Toth, I. K., van der Wolf, J. M., Saddler, G., Lojkowska, E., Helias, V., Pihronen, M., et al. (2011). Dickeya species: an emerging problem for potato production in Europe. Plant Pathol. 60, 385–399. doi: 10.1111/j.1365-3059.2011.02427.x

UniProt Consortium (2015). UniProt: a hub for protein information. Nucleic Acids Res. 43, D204–D212. doi: 10.1093/nar/gku989

Van Dam, P., de Sain, M., ter Horst, A., van der Gragt, M., and Rep, M. (2018). Use of comparative genomics-based markers for discrimination of host specificity in Fusarium oxysporum. Appl. Environ. Microbiol. 84:e01868–17. doi: 10.1128/AEM.01868-17

Vargas, W. A., Martin, J. M. S., Rech, G. E., Rivera, L. P., Benito, E. P., Diaz-Minguez, J. M., et al. (2012). Plant defense mechanisms are activated during biotrophic and necrotrophic development of Colletotrichum graminicola in maize. Plant Physiol. 158, 1342–1358. doi: 10.1104/pp.111.190397

Waldele, E. L. (1945). Comparative studies of some peritrichous phytopathogenic bacteria. Iowa State J. Sci. 19, 435–484.

Waleron, M., Miszat, A., Waleron, M., Franczuk, M., Jonca, J., Wielgomas, B., et al. (2019). Pectobacterium zantedeschiae sp. nov. a new species of a soft rot pathogen isolated from Calla lily (Zantedeschia sp.). Syst. Appl. Microbiol. 42:153. doi: 10.1016/j.syapm.2018.08.004

Waleron, M., Miszat, A., Waleron, M., Franczuk, M., Wielgomas, B., and Waleron, K. (2018). Transfer of Pectobacterium carotovorum subsp. carotovorum strains isolated from potatoes grown at high altitudes to Pectobacterium peruvianum sp. nov. Syst. Appl. Microbiol. 41, 85–93. doi: 10.1016/j.syapm.2017.11.005

Wang, L., X. B., Song, H. C., An, K., Luo, H. M., and Liu, X. J. (2017). Soft rot of potatoes caused by Bacillus amyloquefaciens in Guangdong province China. Can. J. Plant pathol. 39, 533–539. doi: 10.1080/07060 661.2017.1381994

Winnenburg, R., Baldwin, T. K., Urban, M., Rawlings, C., Köhler, J., Hammond-Kosack, K. E., et al. (2006). PHA-base: a new database for pathogen host interactions. Nucleic Acids Res. 34, D459–D464. doi: 10.1093/nar/gkj047

Wirawan, A., Kwoh, C. K., Hsu, L. Y., and Koh, T. H. (2016). “INVERTER: integrated variable number tandem repeat finder,” in Computational Systems-Biology and Bioinformatics. eds. J. H. Chan, Y.-S. Ong and S.-B. Cho (Berlin: Springer), 151–164.

Xie, H., Li, X. Y., Ma, Y. L., and Tian, Y. (2017). First report of Pectobacterium aroidearum causing soft rot of Chinese cabbage in China. Plant Dis. 102:674. doi: 10.1094/PDIS-07-17-1059-PDN

Xie, Y. P., Shao, X. L., and Deng, X. (2019). Regulation of type III secretion system in Pseudomonas syringae. Environ. Microbiol. 21, 4465–4477. doi: 10.1111/1462-2920.14779

Xu, P. D., Wei, D. D., Li, Z. P., Qin, C. X., Li, X., Lin, C. H., et al. (2020). First report of bacterial soft rot on Syngonium podophyllum caused by Pectobacterium aroidearum in China. Plant Dis. 104:2720. doi: 10.1094/ PDIS-03-20-0552-PDN

Yaganza, E. S., Tweddel, R. J., and Arul, J. (2014). Postharvest application of organic and inorganic salts to control potato (Solanum tuberosum L.) storage soft rot: plant tissue-salt physicochemical interactions. J. Agric. Food Chem. 62, 9223–9231. doi: 10.1021/jf5017863

Yang, J. K., Tian, B. Y., Liang, L. M., and Zhang, K. Q. (2007). Extracellular enzymes and the pathogenesis of nematophagous fungi. Appl. Microbiol. Biotechnol. 75, 21–31. doi: 10.1007/s00253-007-0881-4

Zhang, Y., Fan, Q., and Borre, A. (2016). A re-evaluation of the taxonomy of phytopathogenic genera Dickeya and Pectobacterium using whole genome sequencing data. Syst. Appl. Microbiol. 39, 252–259. doi: 10.1016/j.syapm.2016.04.001

Zhang, D., Zhou, Y., Zhao, D. M., Zhou, J. M., Yang, Z. H., and Zhu, M. M. (2017). Complete genome sequence and pathogenic genes analysis of Pectobacterium atrosepticum JG10-08. Genes Genom. 39, 945–955. doi: 10.1007/ s13258-017-0559-y

Zhou, Y., Liang, Y. J., Lynch, K. H., Dennis, J. I., and Wishart, D. S. (2011). PHAST: a fast phage search tool. Nucleic Acids Res. 39(Suppl. 2), W347–W352. doi: 10.1093/nar/gkq485

Zoledowska, S., Motyka-Pomagruk, A., Slodz, W., Mengoni, A., and Lojkowska, E. (2018). High genomic variability in the plant pathogenic bacterium Pectobacterium parmentieri deciphered from de novo assembled complete genomes. BMC Genomics 19:751. doi: 10.1186/s12864-018-5140-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Xu, Wang, Qin, Li, Lin, Liu and Miao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.