Sequencing of the Litchi Downy Blight Pathogen Reveals It Is a *Phytophthora* Species With Downy Mildew-Like Characteristics

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Accepted 10 May 2016.

On the basis of its downy mildew-like morphology, the litchi downy blight pathogen was previously named *Peronophythora litchii*. Recently, however, it was proposed to transfer this pathogen to *Phytophthora* clade 4. To better characterize this unusual oomycete species and important fruit pathogen, we obtained the genome sequence of *Phytophthora litchii* and compared it to those from other oomycete species. *P. litchii* has a small genome with tightly spaced genes. On the basis of a multilocus phylogenetic analysis, the placement of *P. litchii* in the genus *Phytophthora* is strongly supported. Effector proteins predicted included 245 RxLR, 30 necrosis-and-ethylene-inducing protein-like, and 14 crinkler proteins. The typical motifs, phylogenies, and activities of these effectors were typical for a *Phytophthora* species. However, like the genome features of the analyzed downy mildews, *P. litchii* exhibited a streamlined genome with a relatively small number of genes in both core and species-specific protein families. The low GC content and slight codon preferences of *P. litchii* sequences were similar to those of the analyzed downy mildews and a subset of *Phytophthora* species. Taken together, these observations suggest that *P. litchii* is a *Phytophthora* pathogen that is in the process of acquiring downy mildew-like genomic and morphological features. Thus *P. litchii* may provide a novel model for investigating morphological development and genomic adaptation in oomycete pathogens.

Litchi (or lychee; *Litchi chinensis*) is a tropical and subtropical fruit tree extensively grown in China, Southeast Asia, the Indian Subcontinent, and, more recently, South Africa, Brazil, the Caribbean, Queensland, California, and Florida (Crane et al. 2005; Mitra and Pathak 2008; Morton 1987). *Phytophthora litchii*, formerly *Peronophythora litchii* (Chen 1961; Göker et al. 2007), causes significant pre- and post-harvest crop losses due to litchi downy blight disease. *P. litchii* can attack fruit, panicles, and new shoots of litchi, causing panicle rot as well as withering and watery brown spots of fruits (Ann and Ko 1984; Vien et al. 2001; Wang et al. 2010).

P. litchii is an oomycete, a group that shares the kingdom Straminipila with photosynthetic algae such as diatoms (thus, is distinctive from fungi) and that includes many pathogens of plants and animals. However, the precise taxonomy of *P. litchii* has long been ambiguous. It produces *Peronospora*-like sporangia but can grow on artificial media (Supplementary Fig. S1). On the one hand, the differentiated, branched, and determinate sporangia of *P. litchii* suggest a taxonomic affinity with downy mildews such as *Peronospora* species. On the other hand, the mode of zoospore release from the sporangia, the morphology of the sex organs, and the ability to grow on artificial media are consistent with an affinity with *Phytophthora* spp. Due to its transitional features, *P. litchii* was previously named as *Peronophythora litchii* (Chen 1961), and the family Peronophytoraceae, containing only a single genus (*Peronophythora*) and species (*P. litchii*), was subsequently established to accommodate this ambiguous species (Ko et al. 1978). But later, this species was suggested to be a *Phytophthora* species and was renamed as *Phytophthora litchii* according to its morphology during asexual reproduction (Chi et al. 1982; Ho et al. 1984; Huang et al. 1983). More recently, the taxonomic status of *P. litchii* was re-evaluated via phylogenetic analysis of the sequences of large subunit ribosomal DNA sequences (D1 and D2 region), ribosomal intergenic transcribed spacer and 28S sequences, and multiple genes, and nearly all these analyses supported the idea that *P. litchii* is a *Phytophthora* species (Göker et al. 2007; Riethmüller et al. 2002; Voglmayr 2003; Zhang et al. 2007). The most recent reports placed this species in clade 4 of the genus *Phytophthora* (Göker et al. 2007; Kroon et al. 2012).

Although some basic biological characteristics of *P. litchii* have been described, the molecular mechanisms of pathogen development and infection have not been elucidated in this unusual oomycete and important litchi pathogen. Here, we have sequenced the genome of *P. litchii* and compared it to other oomycete genomes. The results reveal a number of genomic characteristics in common with downy mildews as well as many in common with *Phytophthora* species.

RESULTS

P. litchii has a small genome with tightly spaced genes.

Genomic DNA of *P. litchii* was sequenced using Illumina paired-end sequencing technology. A total of 3.2 Gb of clean reads were generated and assembled into 2,543 scaffolds, with a total assembly length of 38 Mb and an N₅₀ scaffold length of

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*The e-Xtra logo stands for “electronic extra” and indicates that 12 supplementary figures and 13 supplementary tables are published online.

35 kb (Supplementary Table S1). The repeated sequence content and genome size were estimated to be 33% and 58 Mb, respectively (Supplementary Fig. S2). The difference between the assembly length and the estimated genome size is likely due to poor assembly of repeat-rich regions. Based on the core eukaryotic genes mapping approach (CEGMA) pipeline, *P. litchii* orthologs could be identified for 97% of the 248 conserved single-copy eukaryotic genes, suggesting that the genome assembly encompassed nearly the complete gene space (Supplementary Fig. S3).

A total of 13,155 gene models were predicted, with an average length of 1,648 bp, 1.6 introns per gene, and an average intergenic space of 1,241 bp within the 38 Mb length of the assembly. The mean gene length and intron number were similar to other sequenced *Phytophthora* genomes, except that of *P. capsici* (Table 1). The estimated repeated sequence content was comparable to many other oomycetes, with the exception of *P. infestans* and *Pythium ultimum* (Table 1). However, the estimated genome size was smaller than most of the analyzed oomycete genomes, except those of *P. capsici* and *Pythium ultimum*, while the gene number and average intergenic space were the smallest of any of the oomycetes (Table 1).

The distributions of intergenic region lengths were compared among the above organisms (Fig. 1). As noted above, genes in the *P. litchii* genome appeared tightly spaced, around 346 genes

per Mb, which was similar to *Pythium ultimum* (Fig. 1A and B) and more closely spaced than the other analyzed *Phytophthora* (Fig. 1C and D) and downy mildew (Fig. 1E through G) genomes.

Low GC content and slight codon preference.

Among 11 oomycete genomes, the G+C content (GC content or %GC) of the *P. litchii* assembly (49%) was the lowest of all the *Phytophthora* genomes but higher than those of the downy mildews (Table 1; Supplementary Fig. S4A). We further compared GC content in gene-coding and non-gene coding regions as well as codon first, second, and third positions of gene-coding regions (%GC1, %GC2, and %GC3, respectively). In general, the coding regions of each species exhibited higher %GC values than those of the noncoding regions. However, across the 11 species, %GC values of coding and noncoding regions exhibited strong positive correlation ($R^2 = 0.79$), indicating that the bias of GC content among the species was genome wide. In addition, %GC values of the coding regions were most correlated with %GC of the codon third position ($R^2 = 0.99$). In nearly all species, the codon third position exhibited the strongest preference for G or C and the largest range of values among the analyzed species. In contrast, the %GC values of the first and second codon positions were slightly higher and much lower than the overall coding regions, respectively.

Table 1. Genome statistics of *Phytophthora litchii* and other oomycetes^a

Categories	<i>Phl</i>	<i>Phs</i>	<i>Phr</i>	<i>Phi</i>	<i>Phc</i>	<i>Pyu</i>	<i>Hya</i>	<i>Pet</i>	<i>Plh</i>	<i>Sap</i>
Estimated genome size (Mb)	58	95	65	240	57	45	99	68	100	63
Assembly size (Mb)	38	86	67	229	64	43	82	63	75	53
G+C content (%)	49	54	54	51	50	52	47	48	45	58
Repeat (%)	33	39	28	74	19	7	35	24	39	40
Number of genes	13,155	19,027	15,743	17,797	19,805	15,297	14,567	18,447	15,469	17,065
Gene length mean (bp)	1,648	1,614	1,624	1,525	1,253	1,503	1,113	1,250	1,143	1,521
Mean intergenic distance (bp)	1,241	2,906	2,632	11,342	1,979	1,308	4,516	2,171	3,705	1,585
Mean introns per gene	1.6	1.6	1.6	1.8	1.2	1.6	1.0	2.3	2.6	3.0

^a *Phl*, *Phytophthora litchii*; *Phs*, *Phytophthora sojae*; *Phr*, *Phytophthora ramorum*; *Phi*, *Phytophthora infestans*; *Phc*, *Phytophthora capsici*; *Pyu*, *Pythium ultimum*; *Hya*, *Hyaloperonospora arabidopsidis*; *Pet*, *Peronospora tabacina*; *Plh*, *Plasmopara halstedii*; and *Sap*, *Saprolegnia parasitica*.

Fig. 1. Distribution of intergenic region lengths in *Phytophthora litchii* compared with other oomycetes. **A**, *Pythium ultimum*, **B**, *P. litchii*, **C**, *P. sojae*, **D**, *P. infestans*, **E**, *Peronospora tabacina*, **F**, *Plasmopara halstedii*, and **G**, *Hyaloperonospora arabidopsidis*. The flanking distance between neighboring genes provides a measurement of local gene density. Values in brackets show the average number of predicted genes per megabase of assembled genome sequences.

Next, we calculated relative synonymous codon usage (RSCU) values for each of the species (Supplementary Table S2). We clustered the patterns of RSCU values according to both codons and species, using hierarchical (HCL) methods (Fig. 2A). Nearly all codons ending with G or C had high RSCU values (greater than 1, indicating that the codon was more frequent than expected; *Plasmopara halstedii* was an exception), indicating a considerable preference for codons ending with G or C (Fig. 2A). Those codons were clustered in a group. Among the species, *P. sojae*, *P. ramorum*, and *Pythium ultimum* formed one cluster, while *P. litchii*, *P. infestans*, *P. parasitica*, *P. capsici*, *Hyaloperonospora arabidopsidis*, and *Peronospora tabacina* formed a second cluster. *Plasmopara halstedii* and *Saprolegnia parasitica* were outliers (Fig. 2A). We also analyzed the patterns of RSCU values among the species, using principal component analysis (PCA) (Fig. 2B). Again, the results revealed that the species exhibited two major groups, according to high versus low GC content and strong versus slight codon preference. The three downy mildews clustered with four of the *Phytophthora* species, namely *P. litchii*, *P. infestans*, *P. parasitica*, and *P. capsici*, with *Plasmopara halstedii* the most outlying member of this cluster. These seven species exhibited low GC content and slight codon preference. *Pythium ultimum*, *P. sojae*, and *P. ramorum* formed a second cluster with higher GC content and stronger bias, while *S. parasitica* remained an outlier (Fig. 2B).

Multigene phylogenetic analysis confirms *P. litchii* is in *Phytophthora* clade 4.

Phylogenetic trees of 12 genome-sequenced oomycete species were constructed, based on 293 core genes identified by the CEGMA pipeline. Both the concatenated DNA sequences of the genes and the concatenated amino acid sequences encoded by the genes were analyzed. Before constructing trees using RaxML, PartitionFinder software was used to select best-fit partitioning schemes and models of molecular evolution for nucleotide and amino acid alignments, respectively. The trees constructed using protein and DNA sequences and considering one single partition and multiple partitions displayed nearly identical topological structures. The downy mildews formed a cluster nested within the *Phytophthora* clade, and *P. litchii* was nested in a subcluster of the *Phytophthora* clade that was close to the downy mildew cluster (Fig. 3A). Consistent with the phylogenies reported in some previous studies (Göker et al. 2007; Riethmüller et al. 2002; Voglmayr 2003), this placement of *P. litchii* added evidence in support of the theory that *P. litchii* is a *Phytophthora* species. Furthermore, a recent phylogenetic analysis of the genus *Phytophthora* (Martin et al. 2014) was repeated, incorporating corresponding sequences from the *P. litchii* genome assembly. Two trees, constructed using five nuclear (Supplementary Fig. S5A) or four mitochondrial loci, supported the idea that *P. litchii* is in clade 4 of the genus *Phytophthora*.

Protein sequence identity and gene colinearity with *Phytophthora* genomes.

In order to evaluate the overall sequence similarity of *P. litchii* proteins with those of other oomycetes and stramenopiles, all 13,155 predicted protein sequences of *P. litchii* were compared with translated genome sequences of the other species, using a TBLASTN search with 1e-8 as the E-value cut-off. The largest numbers of *P. litchii* protein sequences matched the translated genome sequences of the six *Phytophthora* species, while lower matches were observed to those of the other five oomycete species. Even lower numbers of matches were observed to protein sequences from the stramenopile diatom *Phaeodactylum tricornutum* and the fungus *Magnaporthe*

Fig. 2. Clustering of species and codons according to the relative synonymous codon usage (RSCU) values. **A**, Heat map of RSCU values clustered according to species and codons. Red and green colors indicate RSCU values higher and lower than 1, indicating strong and weak preference for codon usage, respectively. # indicates codons that end with G or C. The labels of species were colored according to the clustering results. **B**, Based on RSCU values, the species were clustered using principal components analysis. Principal components 1 and 2 together represent 93.9% of the eigenvalues. Sap, *Saprolegnia parasitica*; Pyu, *Pythium ultimum*; Phr, *P. ramorum*; Phs, *P. sojae*; Phc, *P. capsici*; Php, *P. parasitica*; Phi, *P. infestans*; Plh, *Phytophthora litchii*; Pet, *Peronospora tabacina*; Hya, *Hyaloperonospora arabidopsidis*; Plh, *Plasmopara halstedii*. The colors of labels are the same as those in A.

oryzae (Fig. 3B). Across the spans of the matched regions, the *P. litchii* protein sequences showed a median identity of more than 71% with the corresponding sequences from each *Phytophthora* genome, whereas the median identities between the *P. litchii* and downy mildew sequences ranged from 66 to 69%. Lower median identities were observed for protein sequences from *Pythium ultimum* (56%) and *S. parasitica* (45%) and even lower for *Phaeodactylum tricornutum* and *M. oryzae* (Fig. 3C).

As a comparison, the same TBLASTN search was performed using all predicted protein sequences from *P. sojae* against the genome sequences of *P. infestans* and *P. litchii*. There were similar numbers of matched *P. sojae* proteins and similar median sequence identities with the *P. infestans* and *P. litchii* genomes (Fig. 3B and C), supporting the theory that the sequences of the *P. litchii* proteins are similar to those of the other *Phytophthora* species.

We used DAGchainer to examine gene colinearity between the *P. litchii* genome and the other oomycete genomes. The numbers of *P. litchii* genes showing colinearity were greatest in the comparison with *Phytophthora* species (7,047 and 6,531 with *P. infestans* and *P. sojae*, respectively) and least with *H. arabidopsidis* (3,240) (Supplementary Fig. S6; Supplementary Table S3). There were 678 and 637 blocks of colinearity with the two *Phytophthora* genomes with, respectively, 10.4 and 10.3 genes per block on average. In contrast, there were only 394 blocks of colinearity with the *H. arabidopsidis* genome with 8.2 genes per block. Thus, the genes of *P. litchii* exhibited much greater colinearity with the genes in the *Phytophthora* genomes.

Genes for nitrate, nitrite, and sulfite reduction are retained in *P. litchii*.

In the obligate biotroph *H. arabidopsidis* and the oomycete fish pathogen *S. parasitica*, genes for nitrate and nitrite reductases, a nitrate transporter, and sulfite reductase are absent (Baxter et al. 2010; Jiang et al. 2013). Similar genes have been lost from obligate fungal pathogens (Spanu et al. 2010). In two

recently available downy mildew assemblies, none of the five genes could be found in *Plasmopara halstedii* but, interestingly, two sulfite reductase genes could be identified in *Peronospora tabacina* (Supplementary Table S4; Supplementary Fig. S7A). In contrast to these downy mildews, all five genes could be readily identified from the *P. litchii* genome assembly, as in other *Phytophthora* species. The existence or absence of certain genes was confirmed in the gene colinearity analysis. A phylogenetic analysis of these five *P. litchii* proteins relative to those from other *Phytophthora* species and their most identical homologs from other eukaryotes supported the idea that the *P. litchii* proteins shared a common origin with the other *Phytophthora* proteins.

P. litchii gene families have fewer members than other *Phytophthora* species.

To learn why the *P. litchii* genome had a reduced number of genes, we used TribeMCL (Enright et al. 2003) to cluster the predicted proteins of *P. litchii*, *P. sojae*, *P. infestans*, *H. arabidopsidis*, and *Pythium ultimum* into families (Supplementary Table S5). We discovered that *P. litchii* shared more clustered protein groups (Fig. 4A) and group members (Fig. 4B) with *P. infestans* and *P. sojae* than with *H. arabidopsidis* and *Pythium ultimum*. Approximately 60% of the predicted proteins in each species were clustered into groups that contained at least one member (Fig. 4C). However, the average numbers of proteins per group were different, ranging from 2.69 in *P. sojae* and 2.40 in *P. infestans* to 1.98 in *P. litchii* and 1.91 in *H. arabidopsidis* (Fig. 4D). The clustering also identified variable numbers of proteins that were species-specific. In *P. sojae* and *P. infestans*, 6.5 and 8.7% of the proteins, respectively, were species-specific. In contrast, only 1.11% of the genes in *P. litchii* were species-specific but 22% of the genes in *H. arabidopsidis* were species-specific (Fig. 4E). The average numbers of proteins per group ranged from 1.70 in *P. sojae* and 1.75 in *P. infestans* to 1.11 in *P. litchii* and 1.13 in *H. arabidopsidis* (Fig. 4F).

Fig. 3. Phylogenetic analysis and comparisons of protein sequence identity. **A**, Multisequence phylogenetic tree constructed by RaxML (ML + rapid bootstrap method with 200 bootstrap repeats), using concatenated DNA or protein sequences of 293 core genes identified by CEGMA analysis. The topology of the tree was nearly identical, irrespective of whether the DNA or amino acid sequences were used or whether one single position or multiple positions were evaluated. Numbers near branches denote bootstrap values based on (from left to right): protein sequences, 182 PartitionFinderProtein-suggested positions, LG+G model; protein sequences, one single position, LG+G+F model; DNA sequences, 114 PartitionFinder-suggested positions, GTR+G model; DNA sequences, one single position, GTR+G model. Asterisks indicate 100% bootstrap by all four methods. **B**, The columns show the proportions of *Phytophthora litchii* or *P. sojae* proteins that have TBLASTN hits ($<1e-8$) against the genome of each listed species. **C**, The boxplots show distributions of *P. litchii* or *P. sojae* proteins having different percent identities within TBLASTN alignments with translated genome sequences from each listed species. The bottom and top of the box represent the first and third quartiles and the band inside the box represents the median. The whisker ends represent the minimum and maximum of all data. Phk, *Phytophthora kernoviae*; Phr, *P. ramorum*; Phs, *P. sojae*; Php, *P. parasitica*; Phi, *P. infestans*; Phc, *P. capsici*; Pet, *Peronospora tabacina*; Hya, *Hyaloperonospora arabidopsidis*; Plh, *Plasmopara halstedii*; Pyu, *Pythium ultimum*; Sap, *Saprolegnia parasitica*; Pht, *Phaeodactylum tricornutum*; and Mao, *Magnaporthe oryzae*; and Phl, *Phytophthora litchii*. In A through C, red indicates *Phytophthora* species, green indicates downy mildew species, blue indicates other oomycetes, and gray indicates nonoomycete species.

We also compared the number of proteins mapped into PFAM protein families (Supplementary Table S6). Again, protein numbers per PFAM family in *P. litchii* were generally less than those in *P. sojae*, *P. ramorum*, and *P. infestans* and greater than those in *H. arabidopsidis* and *Plasmopara halstedii* (Supplementary Fig. S8A and B). Since *P. litchii* is a plant pathogen, we further checked the putative pathogenicity-related PFAM families. The majority of these families had fewer or a comparable number of members than in the analyzed *Phytophthora* species but distinctly more members than in the analyzed downy mildews. We analyzed genes encoding potential cell wall-degrading enzymes (CWDE), including carbohydrate esterases, glycoside hydrolases, and polysaccharide lyases, and obtained similar results (Supplementary Table S7). Moreover, we searched for gene families expanded in *P. litchii* compared with other *Phytophthora* species, but no significant examples were found (data not shown). Overall, therefore, the results suggest that widespread reductions in gene family membership and in the number of species-specific genes might explain the fewer total number of genes in *P. litchii*.

Families of virulence effectors.

Phytophthora genomes are characterized by three large families of virulence effectors defined by distinctive sequence

motifs, namely RxLR effectors (hundreds of members per genome), crinkler (CRN) effectors (tens to hundreds of members per genome), and necrosis-and-ethylene-inducing protein-like (NLP) effectors (tens of members per genome) (Haas et al. 2009; Tyler et al. 2006). Smaller numbers of these effector genes occur in downy mildews such as *H. arabidopsidis* (Baxter et al. 2010), *Plasmopara halstedii* (Sharma et al. 2015), and *Peronospora tabacina* (Derevnina et al. 2015). Distant relatives of CRN and NLP effectors can be found in fungi (Joneson et al. 2011) and fungi and bacteria (Gijzen and Nurnberger 2006), respectively, while RxLR effector genes appear specific to *Phytophthora* and downy mildew species—none at all could be found in seven surveyed *Pythium* genomes (Adhikari et al. 2013; Lévesque et al. 2010). In *P. litchii*, we identified candidates for these three effector families, including 245 RxLR (Supplementary Table S8), 30 NLP (Supplementary Table S9), and 14 CRN (Supplementary Table S10) proteins. These candidate effector proteins contained sequence features and motifs characteristic of the respective effector families (Supplementary Fig. S9A to D). The numbers of CRN and NLP effector gene candidates were comparable to those of the other *Phytophthora* species, though at the low end of the range in each case (Supplementary Table S11). The number of RxLR effector candidates was somewhat fewer than the other *Phytophthora* species but almost twofold greater than those of downy mildews, e.g., 134 in *H. arabidopsidis*

Fig. 4. Comparison of oomycete protein groupings and group memberships. Numerals indicate the numbers of **A**, clustered groups or **B**, proteins in a group within a particular subset. The numerals outside the Venn diagrams indicate the total number shared between *Phytophthora litchii* and the indicated species. **C**, Percentage of the predicted proteome of each species that is shared across all analyzed oomycete species. **D**, For each species, the average number of proteins per group is shown for proteins shared across all analyzed oomycete species. **E**, Percentage of the predicted proteome of each species that is specific to that species. **F**, For each species, the average number of proteins per group is shown for proteins specific to that species. Phs, *P. sojae*; Phi, *P. infestans*; Phl, *P. litchii*; Hya, *Hyaloperonospora arabidopsidis*; and Pyu, *Pythium ultimum*.

(Baxter et al. 2010) and 120 in *Peronospora tabacina* (Derevnina et al. 2015).

A total of 265 candidates were originally identified from at least one of the two methods for prediction of RxLR effectors, i.e., hidden Markov model (HMM) and BLASTP (Supplementary Fig. S10A). Regular expression searches were further used to check if the candidates contained one or both “R/Q/GxLR” or “EER” strings at the 21st to 120th amino acids (aa) of protein sequences. All HMM candidates matched this regular expression, while 20 BLASTP candidates were excluded and were, thus, designated “RxLR-like” candidates. The remaining 245 proteins were defined as the *P. litchii* RxLR candidates. As in the RxLR effector family generally (Ye et al. 2015), the predicted secondary structures of these *P. litchii* RxLR proteins exhibited a high content of alpha-helices. In addition, the features of the typical motifs were more similar to those of the other *Phytophthora* RxLR proteins. Over half of the RxLR proteins in both *P. litchii* and other *Phytophthora* species contained both R/Q/GxLR and EER strings, while the proportions in *H. arabidopsidis* and *Plasmopara halstedii* were 35 and 14%, respectively. Moreover, 76% of *P. litchii* RxLR protein sequences contained a perfect RxLR string within the identified RxLR motif. The top five patterns were RFLR, RLLR, RSLR, RHLR, and RRLR, accounting for 82% of those perfect RxLR strings. Those two percentages were comparable to those in *P. sojae* (79 and 78%), *P. ramorum* (71 and 87%), and *P. infestans* (82 and 81%), but different to *H. arabidopsidis* (60 and 70%), and *Plasmopara halstedii* (15 and 54%) (Supplementary Table S12).

We also identified WY-domain effectors in *P. litchii* and obtained 92 candidates, made up of 87 RxLR and five RxLR-like candidates. Based on the same analysis of the other oomycete species, we found at least 89% of the WY-domain effector candidates in *Phytophthora* species contained the R/Q/GxLR and/or EER strings (most were the RxLR effector candidates), compared with only 67% of *H. arabidopsidis* WY-domain proteins, and 50% in the case of *Plasmopara halstedii*. The WY-domain effectors comprised 36% of all *P. litchii* RxLR candidates, which was similar to the other *Phytophthora* RxLR candidates (35 to 45%) but different from those of *H. arabidopsidis* (28%) and *Plasmopara halstedii* (4%). Thus, in line with the previous reports (Baxter et al. 2010; Derevnina et al. 2015; Sharma et al. 2015), we found the RxLR protein sequences of downy mildews exhibited more variability in the typical effector motifs, whereas the *P. litchii* RxLR proteins were more similar to those of the other *Phytophthora* species. As in other *Phytophthora* species (Jiang et al. 2008), the *P. litchii* WY-domain proteins were predicted to contain one to eight such domains in each sequence (Supplementary Fig. S11A), and the lengths of protein sequences were positively correlated with the numbers of WY-domain in each protein.

We identified 14 candidate members of the CRN effector family from the *P. litchii* genome assembly, based on HMM and BLASTP searches. Consistent with the previous reports that only a few CRN effectors contain a predicted signal peptide that can be recognized by the SignalP algorithm (Derevnina et al. 2015; Haas et al. 2009), we found there was only one such protein in *P. litchii*.

To evaluate the predicted NLP proteins encoded in the *P. litchii* genome, we constructed a maximum-likelihood tree, using available sequences from the oomycetes *P. sojae*, *P. litchii*, *H. arabidopsidis*, *Peronospora tabacina*, *Plasmopara halstedii*, and *Pythium ultimum* and the fungi *M. oryzae* and *Fusarium graminearum* (Fig. 5). The *P. litchii* sequences grouped closely with *P. sojae* sequences, in most cases in pairwise relationships and in some cases in small *P. litchii*-specific subclades. By

comparison, the majority of the downy mildew and *Pythium ultimum* NLP sequences formed clades isolated from each other and from the major *Phytophthora* clade. The fungal NLP proteins formed an even more isolated clade.

In *P. sojae*, several NLP proteins exhibit necrosis-inducing activity when transiently expressed in *Nicotiana benthamiana* (Dong et al. 2012). All these NLP proteins are found in certain clusters (necrosis-inducing clusters) of clades in the tree shown in Figure 5, while outside these clusters, several tested *P. sojae* NLP proteins could not induce necrosis. We randomly selected 12 *P. litchii* NLP effector candidates, including seven close to the necrosis-inducing clusters and five far from such clusters, to assay for induction of cell death in *N. benthamiana*. We found three of the first seven *P. litchii* NLP proteins could induce necrosis in *N. benthamiana*, while the other tested NLP proteins could not (Fig. 5; Supplementary Fig. S12). The results indicate that the NLP proteins of *P. litchii* and *P. sojae* may maintain similar functions or activities.

DISCUSSION

P. litchii is a well-known pathogen of litchi. Due to its downy mildew-like morphology, the taxonomic status of *P. litchii* used to be controversial. Furthermore, the genome information and molecular mechanisms of development and infection of this pathogen have not been examined. In this study, we determined the genome sequence of *P. litchii* and compared its phylogeny, genome size, gene number, gene colinearity, sequence identity, GC content, and codon usage, protein family sizes, and effector features to other oomycete species.

Several features of the *P. litchii* genome affirm that the recent transfer of this species to the genus *Phytophthora* is correct. First, multisequence phylogenies of genome-sequenced oomycete species based on 293 core genes unambiguously placed *P. litchii* within genus *Phytophthora*. Similar results were observed in other phylogenies constructed from specific genes. This conclusion is also supported by analysis of global protein sequence identity and of gene colinearity among the sequenced oomycete genomes. A further *Phytophthora*-like feature was the retention of genes for inorganic nitrogen and sulfur assimilation, which have been lost from the indicated downy mildews (partially lost in *Peronospora tabacina*) and *S. parasitica* genomes. Effector candidates of the RxLR, CRN, and NLP families could readily be identified in *P. litchii*, and the numbers of genes in these families and the sequence or motif features of these proteins were closer to those of *Phytophthora* species. In particular, phylogenetic analysis and necrosis-inducing activity assays of the NLP proteins of *P. litchii* showed them to have close affinity with *Phytophthora* NLP and to be distinct from the NLP from downy mildews and *Pythium ultimum*.

Despite the unambiguous identification of *P. litchii* as a *Phytophthora* species, a number of features of the *P. litchii* genome were distinctive and suggest it is subject to some of the evolutionary forces that have shaped downy mildew genomes. For example, the *P. litchii* genome was unexpectedly small in size and contained fewer genes than other sequenced *Phytophthora* genomes. The lower total number of genes appeared to result from smaller gene families and reduced numbers of species-specific genes, rather than the complete elimination of many gene families. The genomes of downy mildew pathogens such as *H. arabidopsidis* and *Plasmopara halstedii* are also streamlined and showed similar reductions in the sizes of gene families. For example, all the *P. litchii*, *H. arabidopsidis*, and *Plasmopara halstedii* genomes contain reduced numbers of CWDE. Reductions in the number of these particular enzymes may reflect adaptations for stealthy biotrophic growth. Both *P. litchii* and downy mildews also share relatively low GC content and mild codon preferences. However the significance of this feature is not clear.

Some other *Phytophthora* species, such as *P. infestans*, also exhibit this relatively low GC content while others, such as *P. sojae*, have a distinctly higher GC content and strong codon preference.

The relationship of the genera of downy mildews, *Phytophthora*, and *Pythium* has been a matter of ongoing debate. Members

of the genera *Phytophthora* and *Pythium* can, in general, be cultured on standard or nonstandard agar media. In contrast, the downy mildews are obligate biotrophs, since they have not, to date, been cultured on any artificial medium. Previously, genera *Phytophthora* and *Pythium* were grouped together in the family

Fig. 5. Phylogeny of candidate necrosis-and-ethylene-inducing protein-like (NLP) effectors. Phylogenetic tree constructed using typical domains (PFAM ID: PF05630.6). The tree was constructed using PhyML (v3.1) implemented in Seaview (v4.4). Bootstrap values greater than 80 are displayed. Red stars indicate NLP proteins that had necrosis-inducing activity, while green stars indicate NLP proteins that cannot induce necrosis.

Pythiaceae (Waterhouse 1973). More recently, however, analysis of large subunit rRNA gene sequences from 50 downy mildew species and 18 *Phytophthora* species suggested monophyly of a clade comprising both the genus *Phytophthora* and the downy mildews (Göker et al. 2007). That analysis and a later one involving the nucleotide sequences of seven genes from 80 *Phytophthora* species and two downy mildews (Runge et al. 2011) concluded that the genus *Phytophthora* was paraphyletic, while the downy mildew representatives were monophyletic and nested within *Phytophthora* clade 4, which contains *P. litchii*. Similar results were obtained in our study. The phylogenetic analysis, based on 293 orthologous protein-coding genes from seven *Phytophthora* species and three downy mildew species, supported the idea that the downy mildew clade was nested within genus *Phytophthora* and also showed that *P. litchii* was the nearest to the downy mildew cluster. The extended multilocus phylogenies of many *Phytophthora* species further confirmed that *P. litchii* is in clade 4 of the genus *Phytophthora*. Thus, it is tempting to suppose that the downy mildew-like morphological and genomic characteristics of *P. litchii* may be due to an ancestor shared with downy mildews within clade 4 and that *P. litchii* may have acquired downy mildew-like genomic and morphological features through a common ancestor with downy mildews. However, the phylogenetic position still might change with the addition of more *Phytophthora* and downy mildew genomes, due to the effect of incomplete taxon sampling; therefore, the phylogenetic data are not yet strong enough to rule out that *P. litchii* could have acquired these characteristics via convergent evolution.

The small genome size of *P. litchii*, including smaller gene family sizes, makes it an attractive model for molecular manipulation and investigation of the molecular mechanisms of development and infection of oomycete pathogens. We have already successfully executed polyethylene glycol-mediated protoplast transformation of *P. litchii* (*unpublished*). We have preliminarily identified experimentally tractable hosts for *P. litchii*, namely *N. benthamiana* and tomato (*Solanum lycopersicum* L.). Previous reports identified litchi as the only natural host of *P. litchii*, but this pathogen was recently found naturally infecting additional plants. For example, *P. litchii* could also cause leaf and stem blight on longan (*Euphoria longana*) seedlings (Ann et al. 2012), thus providing a range of hosts for experimental investigation of host range.

In conclusion, *P. litchii* represents an unusual *Phytophthora* species in which downy mildew-like morphological features have evolved independently from the true downy mildews, and several features of the genome have also acquired downy mildew-like characteristics. Its compact genome as well as its morphological and physiological features make *P. litchii* a novel potential model species with which to assess the environmental and host adaptability of oomycete pathogens during evolution.

MATERIALS AND METHODS

Genome sequencing and assembly.

The sequenced *P. litchii* strain SHS3 was isolated from Guangzhou Province, China. High-quality genomic DNA was prepared and submitted to the Beijing Genomics Institute for Illumina sequencing. Paired-end reads of 90 bp × 2 in length and 500 bp in insert size were generated. A total of 3,404 Mb of raw data, including 3,205 Mb of clean data, were obtained. The short reads were assembled into a genome sequence using SOAPdenovo (version 1.05). The key parameter, K, was 27. Assembled scaffolds from SOAPdenovo were further linked using SSPACE (version 1.1). Gap filling and single base corrections were performed via SOAPaligner (version 2.21). The final assembly was 38.2 Mb in size and contained 2,543 scaffolds longer than 1,000 bp. The difference between the

assembly size and the estimated genome size (58 Mb) likely results from poor assembly of the repeated sequences that comprise 33% of the genome. The CEGMA pipeline was used to compare the completeness and continuity of the genome (the Korf Lab, University of California-Davis). The sequence reads and assemblies have been deposited in GenBank (Bioproject ID: PRJNA290406).

Gene annotation.

Gene models were predicted from the assembly results using Augustus, trained with the 26,584 gene models of *P. sojae* (Joint Genome Institute [JGI] *Phytophthora sojae* v3.0 database). To classify protein families and identify CWDE, we used HMMER3 to align the predicted protein sequences with HMMs for PFAM families (EMBL-EBI Pfam database) and from the Carbohydrate-active enzyme ANnotation (dbCAN) database. An E-value <0.001 was used for PFAM mapping. In dbCAN mapping, the following criteria were used: if alignment length >80 aa, use E-value <1e-5, otherwise use E-value <1e-3 and covered fraction >0.3. Covered fraction was calculated as (End – Start) divided by length of HMM.

To identify candidate effector proteins, the *P. litchii* genome sequences were translated to all possible open reading frames (ORF) that encoded 80 to 1,000 aa from both strands.

For identification of RxLR candidates, two methods were then used to search these ORF-coding proteins: i) a series of iterative HMM searches (score >0). The initial HMM profile was created on the basis of the *P. sojae* RxLR sequences, while subsequent searches used an HMM profile built from the novel sets of *P. litchii* RxLR candidates. The HMM profiles were built using the aligned protein sequences of RxLR or RxLR-EER motifs along with 5 to 10 aa before and after these motifs. Sequences were aligned using MAFFT (v7.221). And ii) a series of iterative BLASTP searches (E-value <1e-5), using available RxLR proteins from *P. sojae*, *P. ramorum*, *P. infestans*, and *H. arabidopsidis* as the first set of query sequences and the new identified *P. litchii* candidates as the next sets of query sequences. The candidates from i and ii were then checked to verify if they contained one or both R/Q/GxLR or EER strings within the region from the 21st to 120th residues of protein sequences.

For identification of WY-domain proteins and CRN candidates, we performed similar HMM searches, using an alignment of the available WY-domain or full-length CRN sequences from *P. sojae*, *P. ramorum*, and *P. infestans* (Boutemy et al. 2011; Haas et al. 2009). The HMM alignments for the newly identified CRN candidates were manually checked to see whether they contained typical LQLFLAK and HVLVVVP motifs.

For identification of NLP candidates, we conducted a BLASTP search, using the *P. sojae* NLP proteins as query sequences (Dong et al. 2012). The candidates were then manually checked to see if they contained typical GHRHDWE or -like motifs.

Candidate RxLR and NLP effectors but not CRN effectors (because many CRN candidates do not contain a signal peptide that is recognized by SignalP 3.0) (Haas et al. 2009) were also required to contain a predicted signal peptide, based on a threshold HMM score of >0.9 from SignalP 3.0 and to not contain a transmembrane domain (TMHMM 2.0) outside the signal peptide. Sequences of typical motifs were aligned using MAFFT (v7.221) and features of these motifs were illustrated using Weblogo.

Comparative genomic analysis.

Sequences of *P. sojae* (v1.1) (Tyler et al. 2006), *P. ramorum* (v1.1) (Tyler et al. 2006), *P. capsici* (LT1534 v11.0) (Lamour et al. 2012), and *Phaeodactylum tricornutum* (v2.051706) (Bowler et al. 2008) were obtained from the Department of Energy JGI database; *P. infestans* (T30-4) (Haas et al. 2009),

P. parasitica (INRA-310), *S. parasitica* (CBS 223.65) (Jiang et al. 2013), and *M. oryzae* (MG8) (Dean et al. 2005) were obtained from the Broad Institute database; *P. kernoviae* (AOFI00000000.2) (Sambles et al. 2015) were obtained from the GenBank database; *H. arabidopsidis* (v8.3) (Baxter et al. 2010) were obtained from the Eumicrobedb database; *Plasmodium halstedii* were obtained from M. Thines (IPF, Bik-F, Senckenberg, Frankfurt, Germany) (Sharma et al. 2015); *Peronospora tabacina* (968-J2) were from the National Center for Biotechnology Information (BioProject: PRJNA285243) (Derevnina et al. 2015); *Pythium ultimum* (DAOM BR144, pug1) were obtained from the *Pythium* genome database (Lévesque et al. 2010).

Gene colinearity was analyzed using DAGchainer. As input into DAGchainer, BLASTP comparisons were performed using $1e-5$ as a cut-off E-value to identify candidate ortholog pairs. DAGchainer uses a list of gene pairs along with the coordinates of the gene pairs and the protein sequence similarity between a protein pair to identify chains of genes with maximal sequence similarity and identical gene order. By optimizing a score comprised of both colinearity and sequence similarity, DAGchainer identifies protein pairs most likely to be true orthologs. BLASTP comparisons using $1e-5$ as an E-value cut-off were also used as input into TribeMCL for clustering. Protein sequence clusters with members from only a single species were defined as species-specific proteins. Percent GC and percent GC3 contents in genome and gene-coding sequences were calculated using scripts. RSCU values were calculated using the CodonW program. Creation of heat maps and HCL and PCA clustering were done using MultiExperiment Viewer (MEV v4.7.4).

Phylogenetic analysis.

The multigene phylogenetic trees were conducted based on the concatenated sequences of core genes identified by the CEGMA pipeline. The 293 core genes found in all 12 analyzed genomes were first aligned one by one using MAFFT (v7.221) and its “L-INS-I” and “auto” parameters for protein and DNA sequences, respectively. PartitionFinder (v1.1.1) was used to select best-fit partitioning schemes and models of molecular evolution for nucleotide and aa alignments, respectively. Based on the obtained results, we performed RaxML (v8.1.21) to construct trees, using the ML + rapid bootstrap method (–f a) and 200 bootstrap repeats. *S. parasitica* was set as outgroup. A similar method was used to construct the mitochondrial and nuclear multilocus phylogenetic trees for a larger number of *Phytophthora* species. *Pythium ultimum* was set as outgroup. The other phylogenetic trees were constructed by the neighbor-joining method, using MEGA software (v5.10) or by the maximum-likelihood method using PHYML implemented in Seaview (v4.4). All of the trees were viewed using MEGA.

Transient expression of NLP proteins in *N. benthamiana*.

Full-length NLP genes, including the signal peptide coding region, were amplified from *P. litchii* or *P. sojae* genomic DNA by polymerase chain reaction (PCR), using high-fidelity DNA polymerase (TaKaRa Inc., Dalian, China) with the indicated primers (Supplementary Table S13). The products were then cloned into PVX vector pGR107. A 3xHA (hemagglutinin) tag was added onto the C-terminus of the NLP coding regions. The constructs were confirmed by sequencing. Recombinant plasmids were maintained and propagated in *Escherichia coli* JM109, and then, were introduced into *Agrobacterium tumefaciens* GV3101, using electroporation. *A. tumefaciens* transformants were grown at 30°C on Luria Bertani (LB) agar plates supplemented with kanamycin at 50 µg/ml for 2 days. Colonies

were verified by PCR, using vector primers. For infiltration into leaves, recombinant strains of *A. tumefaciens* were grown in LB media supplemented with kanamycin at 50 µg/ml for 1 day at 30°C, with constant shaking. The cells were harvested by centrifugation at $5,000 \times g$ for 3 min, and were then washed three times with 1 ml of induction medium (10 mM MES, 10 mM MgCl₂, and 150 mM acetosyringone, pH 5.6). The *A. tumefaciens* cells were resuspended in induction medium to a final optical density at 600 nm of 0.4 and were then incubated at room temperature for 3 h, prior to infiltration.

N. benthamiana plants were grown in the greenhouse for 4 to 6 weeks at 25°C during the day time (16 h) and 16°C at night (8 h). The leaves were infiltrated with an *A. tumefaciens* cell suspension through a syringe without a needle. *A. tumefaciens* strains harboring green fluorescent protein and *PsNLP1* were used as negative and positive controls, respectively. Photos were taken 4 days postinoculation.

For Western blot analysis, *N. benthamiana* total proteins were extracted 48 h after inoculation, by grinding 400 mg of leaf tissue in 1 ml of extraction buffer (50 mM Tris, pH 7.4; 150 mM NaCl; and 1% Triton X-100) in the presence of 5 µl of protease inhibitor cocktail (P9599; Sigma-Aldrich, St. Louis). Total protein extract samples were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels using equivalent amounts of protein for each sample in an experiment. After electrophoresis, proteins were transferred from gels to polyvinylidene difluoride membranes. For Western blotting, mouse anti-HA monoclonal antibody (Sigma-Aldrich) and goat antimouse immunoglobulin G-peroxidase conjugate (Sigma-Aldrich) were used as the primary and secondary antibodies, respectively. The membrane was treated with chemiluminescent peroxidase substrate 1 (Thermo Scientific Pierce, Rockford, IL, U.S.A.) for 2 min. The membrane was briefly drained and exposed to BioMax (Kodak, Rochester, NY, U.S.A.) light film several times (depending on results) for exposure signal development.

ACKNOWLEDGMENTS

This work was supported by grants to Y. Wang from the Special Fund for Agro Scientific Research in the Public Interest (201303018), China National Funds for Distinguished Young Scientists (31225022), and the International Science & Technology Cooperation Program of China (2013DFG32030), by grants to W. Ye from the National Natural Science Foundation of China (31401688) and the Natural Science Foundation of Jiangsu Province (BK20140698), and by funding from Oregon State University to B. M. Tyler.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Augustus gene prediction database:
<http://bioinf.uni-greifswald.de/augustus>
 Broad Institute database: <http://www.broadinstitute.org>
 CodonW program: <http://codonw.sourceforge.net>
 DAGchainer software: <http://dagchainer.sourceforge.net>
 dbCAN database: <http://csbl.bmb.uga.edu/dbCAN>
 Department of Energy Joint Genome Institute (JGI) database:
<http://genome.jgi.doe.gov>
 EMBL-EBI Pfam database: <http://pfam.xfam.org>
 Eumicrobedb database: <http://eumicrobedb.org>
 HMMER3 software: <http://hmmer.janelia.org>
 JGI *Phytophthora sojae* v3.0 portal: <http://genome.jgi.doe.gov/Physo3>
 The Korf Lab, University of California-Davis: <http://korflab.ucdavis.edu>
 MAFFT multiple sequence alignment program:
<http://mafft.cbrc.jp/alignment/software>
 MEGA software: <http://megasoftware.net>
 MultiExperiment Viewer: <http://www.tm4.org/mev.html>
 National Center for Biotechnology Information GenBank database:
<http://www.ncbi.nlm.nih.gov/nucleotide>
Pythium genome database: <http://pythium.plantbiology.msu.edu>
 Seaview graphical user interface:
<http://pbil.univ-lyon1.fr/software/seaview.html>
 SignalP 3.0 server: <http://www.cbs.dtu.dk/services/SignalP-3.0>
 SOAPaligner software: <http://soap.genomics.org.cn/soapaligner.html>
 SOAPdenovo assembly method:
<http://soap.genomics.org.cn/soapdenovo.html>
 SSPACE bioinformatic solutions:
<http://www.baseclear.com/bioinformatics-tools>
 TMHMM 2.0 server: <http://www.cbs.dtu.dk/services/TMHMM>
 Weblogo website: <http://weblogo.threeplusone.com>