The phylogeny of Pythium, Phytophthora, and Phytopythium genera

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Oomycetes are eukaryotic microorganisms found in diverse environments and despite resembling fungi species, modern molecular techniques revealed that these microorganisms are more closely related to algae. Their impact on agriculture is very significant, causing disease in a vast variety of hosts. In soybean crops, this group of plant pathogens can result in significant economic losses. Facing the threat that these pathogens pose to soybean production, the present study focused on investigating the frequency, distribution, and phylogenetic relationships of Oomycetes affecting soybeans. In 2023, Oomycete species were retrieved from 284 soil samples collected across the state of Wisconsin. Using internal transcribed spacer (ITS) regions 4 and 6, DNA SANGER sequencing was conducted to identify the species retrieved and construct phylogenetic trees. Both neighbor-joining and maximum likelihood methods were deployed, using the software for statistical analysis RStudio and the software for phylogenetic inference IQ-Tree. The survey resulted in 28 distinct species identified using BLAST. Both methods yielded similar and reliable trees. However, the tree generated using IQ-Tree provided a more comprehensive analysis of the model and tree selection processes. This study illuminates the diversity and distribution of Oomycetes affecting soybeans, offering insights crucial for disease management strategies.

INTRODUCTION

Oomycetes are eukaryotic and ubiquitous organisms living in terrestrial and aquatic environments (Judelson, 2012; Guo et al. 2017). These organisms have nutrition modes, appearance, lifestyle, and production of thread-like filamentous hyphae very similar to Fungi (Beakes et al. 2012; Richards et al. 2006; Tyler 2007; Cavalier-Smith, Chao, 2006). Both fungi and oomycetes operate crucial roles in nutrient cycling, are remarkable pathogens of plants, and show visible similarity in forming colonies of branched hyphae inside their hosts (Money, Davis, Ravishankar, 2004).

However, modern molecular techniques revealed different evolutionary origins (Legeay et al. 2019; Bertier et al. 2013; Lévesque, 2011), and despite the Fungi resemblance, Oomycetes are a member of the *Straminipila* and are closer related to brown algae and diatoms (Adhikari et al., 2013). Additionally, to the molecular distinction, Oomycetes also differ from Fungi (kingdom Eumycota) by presenting cellulose and β-glucans in their cell walls, having coenocytic hyphae, producing biflagellate zoospores, and having diploid vegetative state (Schroeder et al., 2013).

These microorganisms have evolved in either saprophytic or pathogenic lifestyles (Phillips et al., 2008). However, it is known that around 60% of the species are considered pathogenic and

can be considered generalist or specialist, depending on the host range of the specie (Gahagan et al., 2023).

This group can be divided into three clusters with distinct host-interactions behavior: the obligate biotrophs, lacking important functional annotations with an overall metabolism reduction with increased reliance on their host for their growth and survival. The *Saprolegniaceae*, which consists of saprophytic free-living microorganisms and shows the presence of steroid biosynthesis pathways. And lastly, the third group constituted mainly of plant pathogens, is divided into two subclusters, the first containing Pythium and *Globisporangium* species, with biosynthetic pathways that other oomycetes lacked, resulting in a most likely facultative lifestyle. And the second sub-cluster, containing Phytophthora species, consisted of a hemibiotroph group that shows a significant reduction not as extensive as in the obligate parasites (Gómez-Pérez, Kemen, 2021; Margulis, Schwartz, 2000).

The growth of these microorganisms is based on the ramifying hyphae within the host and their sporangia formation on plant surfaces, which later germinate developing zoospores or extending germ tubes. This dynamic is highly influenced by the environment and the presence of humidity, light, adequate temperature, and chemical signals (Xiang, Judelson, 2014).

Both sporangia and zoospores are the main modes of propagation, and for plant pathogenic species, the most important means of originating infections (Hardham, 2009). Furthermore, sexual reproduction is considered a crucial feature for these microorganisms, conferring broad genetic variation and consequently enhanced fitness such as more adapted lineages with increased pathogenicity and aggressiveness (Judelson, 2009; Gavino et al., 2000).

More than 500 different Oomycetes have been reported previously in the literature and many of these organisms are known to cause damage to native plants and significant economic losses in aquaculture and agriculture (Walker, Van West, 2007). In agriculture, diseases caused by Oomycetes are considered a major challenge throughout the world and can cause massive losses in a vast number of crops, including foliar diseases such as late blight on potatoes, blue mold on tobacco, grape downy mildew, plus damping off in a wide range of crops, including soybeans (Cohen and Coffey, 1986; Sharma et al. 2021; Zheng et al. 2016; Chepsergon, Moleleki, 2023; Kamoun et al., 2015; Martin, Blair, Coffey, 2014).

Given the significant impact of Oomycetes on plants, particularly the plant pathogenic genera Pythium, Phytophthora, and Phytopythium, and their role in causing substantial

reductions in yield and quality across various host species, this study aimed to investigate the phylogenetic relationships of soil-borne Oomycete species collected in Wisconsin in 2022. This was accomplished using two phylogenetic inference methods: neighbor-joining and maximum likelihood.

MATERIAL AND METHODS

In 2023, we received 284 soil samples collected in 2022 from soybean fields in 39 different counties throughout the state of Wisconsin. These samples were subjected to the soybean leaf discs technique to bait the Oomycete species present in the soil, the technique consists of exposing these microorganisms to favorable environmental conditions of high-water content and high temperatures to induce zoospore germination.

At the end of two weeks, once the zoospores were germinated, we transferred them to semi-selective media (PARB-H) using soybean leaf discs, the growth presented by the 320 isolates retrieved was then re-transferred to lima bean broth for further mycelial harvesting, resulting in the DNA extraction of 240 samples. The extracted DNA samples were sent to Sanger sequencing of the internal transcriber space (ITS) regions of the microorganism ribosomal DNA (rDNA) using the primers ITS6 (forward) and ITS4 (reverse).

The 480 sequenced files (ab1 format) were uploaded to a folder created on the licensed software Geneious Prime (Version 2024.0.2, GraphPad Software LLC d.b.a Geneious). Previously to assembling, the nucleotides presenting quality inferior to the threshold 0.05 of error probability were trimmed from the 3' and 5' edges using the modified-Mott. This algorithm is based on quality and operates by subtracting the base error probability from an error probability cutoff value (default 0.05) to form the base score. The base error probability is calculated from the quality score (Q), such that $P(error)=10^{(Q/-10)}$ (Richard Mott, personal communication).

Succeeding the reads trimming, we assembled the forward and reverse sequences using the assembler TadPole (https://jgi.doe.gov/data-and-tools/bbtools/). Briefly, Tadpole is a Kmer-based assembler with some additional features, such as error correction and extending reads. This assembler considers each contig as unique kmers - subsequences of length K from the sequence - in a way that the contigs will not overlap by more than K-1 bases.

After the assembly, we then proceeded to identify the species of Oomycetes retrieved using the Basic Local Alignment Search Tool (BLAST+) (Version + 2.15.0, National Center for

Biotechnology Information - NCBI), a widely used tool for comparing nucleotide or protein sequences to sequence databases and calculating the statistical significances of matches (Altschul et al., 1990).

Next, the assembled and identified sequences were selected for further multiple sequence alignment using the commonly used Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm. MUSCLE uses a pairwise profile alignment approach to create multiple alignments of biological sequences in an extremely fast distance estimation using kmer counting and progressive alignment using the log-expectation score, its high speed, and accuracy enable assessment of downstream analysis, like phylogenetic trees and predicted structures (Edgar, 2004a; Edgar, 2004b; Nuin et al., 2006).

With the fasta file from the alignment described above we used two different approaches to infer the oomycete phylogeny: (1) the distance-based and (2) maximum likelihood. For the distance-based method, we performed a neighbor-joining tree search using the 'ape' package in the software RStudio statistical computing (Version 2023.09.1 + 494, R Development Core Team 2018).

The distance was calculated using Tamura and Nei's (1993) model which has four frequency parameters and estimates the number of transitional and transversional substitutions per site, as well as the total number of nucleotide substitutions. Tamura and Nei's model is an accurate and reasonable method that can be applied to large datasets. Once the distance was calculated we proceeded to create the phylogenetic tree.

For the maximum likelihood inference from the aligned sequence, we used IQ-TREE multicore (version 2.3.2 COVID edition, Nguyen et al., 2015) to calculate the site-wise likelihood scores for the best-fitting tree. Maximum likelihood involves the estimation of substitution model parameters and branch lengths on a fixed tree. In this context, IQ-TREE deploys elements of hill-climbing algorithms, random perturbation of current best trees, and a broad sampling of initial trees, such features increase substantially the quality of the tree found and the size of the samples to be analyzed.

Finally, both tree sets obtained from the two inference methods in the current project were generated using the package 'ape' in the software RStudio, version 2023.09.1 + 494. The tips titles were shown for every taxon. For the IQ-TREE output, the nex.iqtree file was used for further

plotting. Additionally, the trees were rerooted using one outgroup species collected within the samples (*Mortirella elongata*) as a reference using the software RStudio.

RESULTS AND DISCUSSION

To compile the two phylogenetic inferences, we identified 245 sequences from different isolates and identified 28 different species from the available reference genes from NCBI. However, due to low-quality issues of the sequences or small-sized reads, we were not able to assemble 139 sequence reads. While these sequences were identified and utilized for frequency analysis, they were ultimately discarded from our alignment and phylogenetic inferences. The species retrieved and the quantity collected of each species are described in Table 1.

Table 1. Species, number of the isolates retrieved and identified from Wisconsin soil samples, and the number of assembled sequences read used for further multiple sequence alignment and phylogeny inference.

Species	Isolates identified	Isolates used in the alignment
Pythium nodosum	21	5
Pythium irregulare	96	38
Pythium conidiophorum	8	2
Pythium torulosum	16	6
Pythium inflatum	9	5
Pythium acanthophoron	19	11
Pythium perplexum	16	5
Pythium myriotylum	2	1
Pythium hypogynum	3	2
Pythium vanterpoolii	9	5
Pythium sylvaticum	2	2
Pythium aristoporum	2	0
Pythium yorkense	3	1
Pythium Tracheiphilum	2	0
Pythium rostratifingens	2	0
Pythium aphanidermatum	1	1
Pythium apiculatum	1	1
Pythium dissotocum	1	1
Pythium graminicola	1	1
Pythium intermedium	1	1
Pythium middletonii	2	2
Pythium ultimum	6	4
Phytophthora sojae	4	1
Phytophthora sansomeana	5	3
Phytopythium vexans	8	4
Phytopythium litorale	1	0
Alternaria alternata	2	2
Clonostachys rosea	1	1
Mortierella elongata	1	1

We predominantly retrieved the Oomycete genera Pythium, Phytophthora, and Phytopythium. Among these, we highlight the frequency of the Pythium species *P. irregulare*, *P. nodosum*, *P. acanthophoron*, *P. torolusum*, *P. perplexum*, *P. vanterpoolii*, and *P. inflatum*. The frequency of the genus Phytophythium was lower when compared to Pythium, followed by the genus Phytophthora as illustrated in Figure 1.

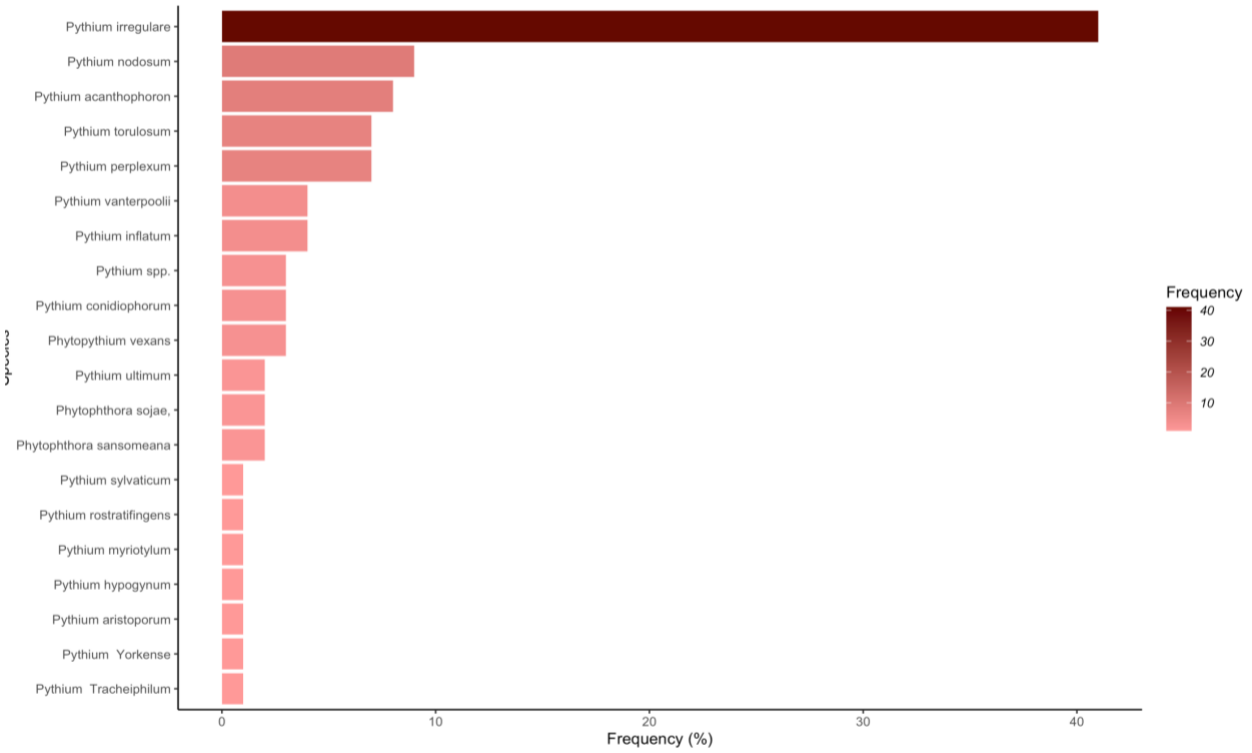

Figure 1. The frequency at which different oomycete species were recovered from Wisconsin soil samples collected in 2022.

Furthermore, we retrieved and identified two *Alternaria alternata* isolates, one isolate *of Clonostachys rosea*, and *Mortierella elongata*. While these species aren't classified as Oomycete, we mantained the sequences, and the reads were included in the alignment (Table 1). In the current project, we used the specie *Mortierella elongata* as our reference outgroup. The selection of this fungal species was primarily because it serves as a suitable outgroup due to its phylogenetic position when compared to the Pythium and Phytophthora genera.

One hundred and six assembled and identified sequence reads were efficiently aligned using the MUSCLE algorithm (Table 1) and yielded a 1,399 bp aligned file that was later used for

the inferences. Facing the small size of the sequences, the small set, and the fast speed characteristic of the MUSCLE algorithm the whole operation lasted for 32 minutes and 22 seconds.

The program MUSCLE is remarkable for its speed processing alignments in big data sets, displaying a high accuracy of the alignment and a substantially improved speed when compared to other software available and widely used, such as CLUSTALW (Edgar 2004a, Edgar 2004b). A more recent comparison of different multiple-sequence alignment software highlighted the speed advantage of MUSCLE when compared to eight other programs. However, MUSCLE showed the disadvantage of being the only program presenting an instability of accuracy, showing sometimes a higher and other times a lower accuracy (Pais et al., 2014). Additionally, the author emphasizes the MUSCLE limitation of not considering which amino acids are occurring between sequences.

The neighbor-joining method using Tamura and Nei's distance calculation of the aligned sequence yielded a tree with 211 nodes and 106 tips (Figure 2). Although this method is simple, computationally inexpensive, doesn't require the download and installation of additional software, and can be performed using the RStudio package 'ape', it did not present sufficient information regarding the methods parameters and the outputs for the tree yielded.

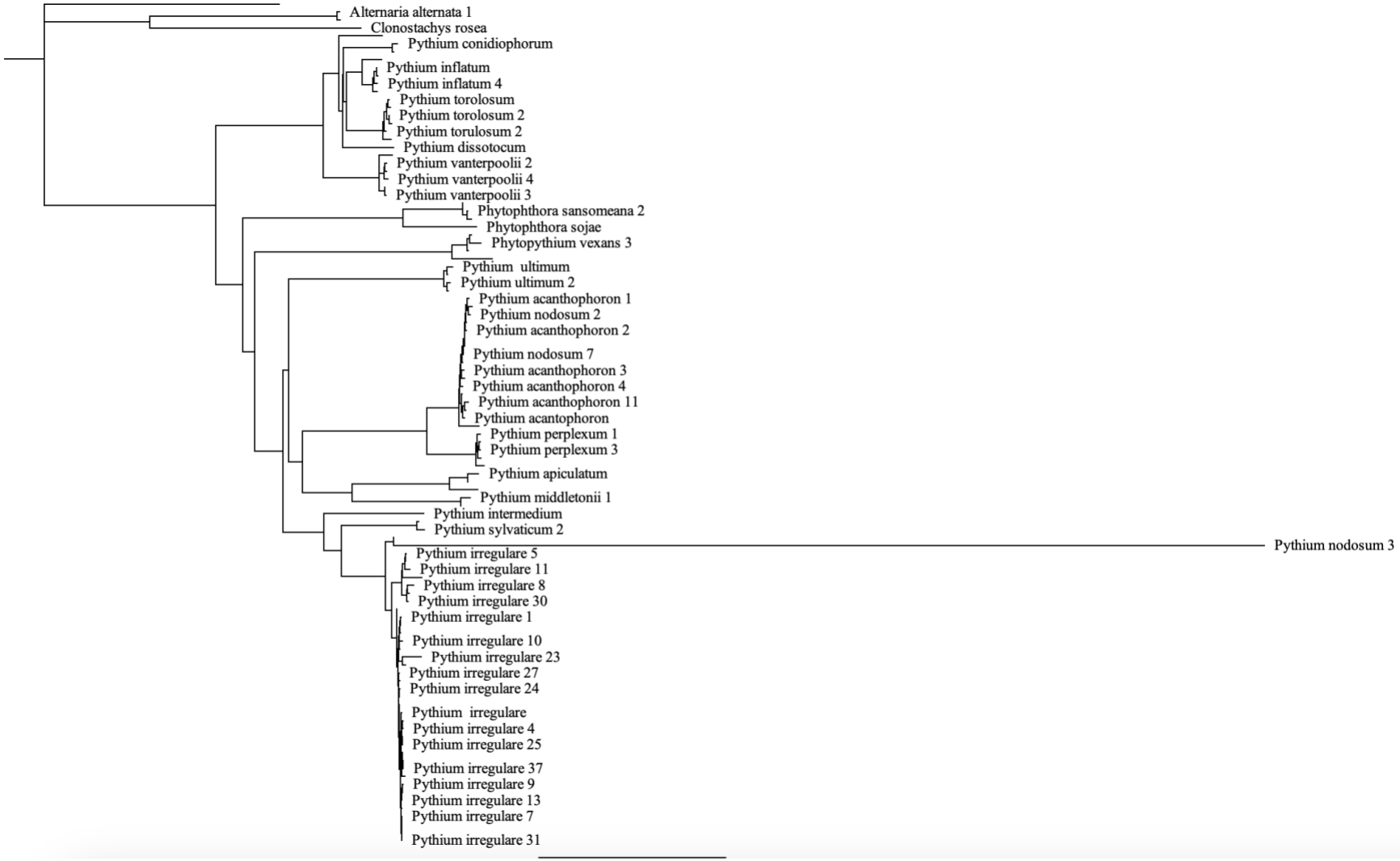

Figure 2. Neighbor-joining phylogeny of the transcribed spacer sequences of the rDNA for oomycete species found during the survey.

Regardless, neighbor-joining (NJ) trees present the advantage of giving a more reliable estimate of the estimate of the evolutionary tree (Edgar, 2004a). NJ is also considered a consistent method that can yield correct trees even when the distance calculated is slightly mistaken (Bruno, 2000). However, NJ often reduces the distance information, leading to data loss, and can only display one possible tree (Amelia Harrision, personal communication).

On the other hand, we use the software IQ-TREE for the inference of a maximum likelihood tree (Figure 3). The current software presented a comprehensive report about the tree generated. The model executed 560 interactions in 492.247 sec (8min:22s) CPU time producing a tree of 11.156 total length. The optimum likelihood was registered at -14427.901, the best fit model was TN+F+I+R5 chosen according to BIC. The base frequencies of A: 0.208, C: 0.193, G: 0.277, and T: 0.322.

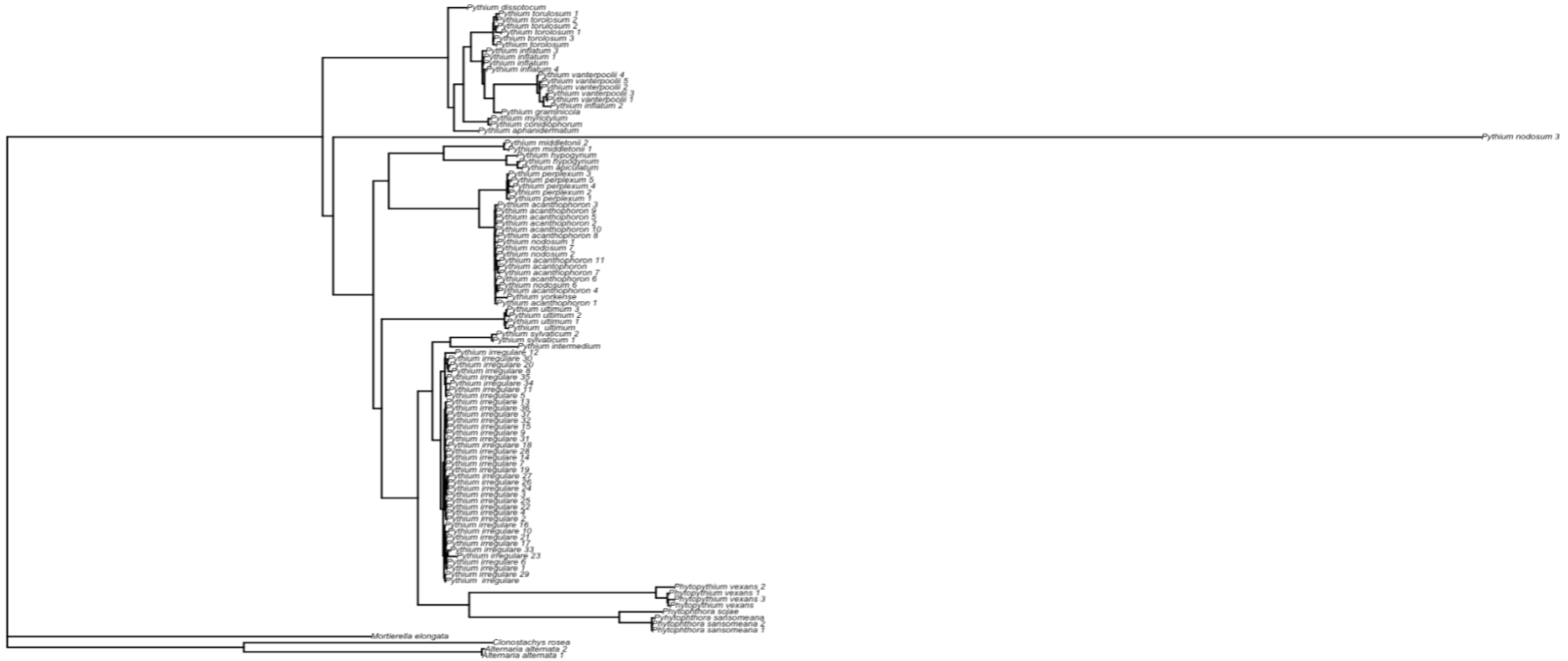

Figure 3. Maximum likelihood phylogeny of the transcribed spacer sequences of the rDNA for oomycete species found during the survey.

The program IQ-TREE is a fast and effective tree searcher that combines renowned phylogenetic and combinatorial optimization techniques. The strategies deployed help to escape local optima and lead to trees with a high likelihood. Besides, the phylogenetic likelihood library reduces de time for likelihood computation. Thus, facing the implementation of hill-climbing and stochastic NNI operations, IQ-TREE can create trees with a higher likelihood than RAxML or PhyML (Nguyen et al., 2015).

In disagreement, Zhou et al. (2018) reported that the use of NNI by IQ-TREE can be disadvantageous when compared to the SPR topological mechanism used by RAxML and PhyML since SPR can explore a greater proportion of tree space than NNI. Therefore, whereas the use of IQ-TREE in smaller sequence datasets (fewer taxa), the use of NNI by this program can show limitations as the data set grows larger.

From a biological perspective, both trees produced very similar outcomes. The fungal species clustered together on a separate and distant branch (Figures 2 and 3). These findings were anticipated, as they belong to entirely distinct organisms. Furthermore, the closer clustering of these microorganisms with *Mortierella elongata* highlights the effectiveness of selecting it as the reference.

For the Oomycete species, we observed the clustering of various isolates of the same species together, driven by their similarity and shared species traits separated in different clades. Hence, this outcome was already expected as well. For both trees, we see the clustering of the species from the genus Pythium in very similar clades.

On the other hand, in the genus Phytopythium, microorganisms that fall in between Phytophthora and Pythium (McCarthy and Fitzpatrick, 2017), were in a separate branch and were represented closer to Phytophthora by IQ-Tree (Figure 3). This result by IQ-Tree is very similar to the tree inferred by Rojas et al. (2017) using the maximum likelihood method.

Overall, both inference methods presented plausible trees for the plant pathogenic Oomycete species sequenced in the present project. Nevertheless, the inclusion of additional sequences would enhance the accuracy of the inferences made, allowing us to further illustrate the key similarities among these species.

CONCLUSION

In the present project, both Neighbor-Joining and Maximum Likelihood inference methods yielded two different trees. However, despite the distinctions in the methods applied, the resulting phylogenetic trees exhibited substantial similarity. Although both trees generated similar inferences, the use of IQ-Tree resulted in a more comprehensive report on the model and tree selection process. Furthermore, the tree generated with IQ-Tree closely resembled previously reported trees, adding to its credibility and consistency.

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