

Phytophthora-ID.org: A Sequence-Based *Phytophthora* Identification Tool

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Abstract

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Contemporary species identification relies strongly on sequence-based identification, yet resources for identification of many fungal and oomycete pathogens are rare. We developed two web-based, searchable databases for rapid identification of *Phytophthora* spp. based on sequencing of the internal transcribed spacer (ITS) or the cytochrome oxidase (*cox*) 1 and 2 spacer region, followed by BLAST searching the databases. Both databases are highly selective. For ITS, only sequences associated with published *Phytophthora* spp. descriptions or classic *Phytophthora* phylogenetics references are included. For the *cox*

spacer region, only data obtained by resequencing select isolates reported in published work were included. Novel taxa tentatively named are selectively included in the database and labeled as *Phytophthora* taxon “X”; as in, for example, *P.* taxon “asparagi”. The database was validated with 700 *Phytophthora* isolates collected from nursery environments during 2006 to 2009. This resource, found at www.Phytophthora-ID.org, is a robust and validated tool for molecular identification of *Phytophthora* spp. and is regularly being updated.

Contemporary species identification relies strongly on sequence-based identification, yet resources for identifying many fungal and oomycete pathogens are rare. The genus *Phytophthora* houses some of the most destructive plant pathogens known, including, for example, the potato late blight pathogen *Phytophthora infestans* (10,11); the sudden oak death pathogen *P. ramorum* (17,31); and *P. cinnamomi*, a pathogen affecting in excess of 3,000 species of plants, including over 2,500 Australian native species and crops such as avocado, pineapple, peach, chestnut, and macadamia (19). Since the discovery of *P. ramorum* in the late 1990s, renewed interest in *Phytophthora* biology has led to the discovery of many new *Phytophthora* spp. In 1996, approximately 60 species of *Phytophthora* were known to science (7). By 2008, around 90 species of *Phytophthora* were described or proposed. In fact, Brasier (3) estimated that we might expect two to four times that number of *Phytophthora* spp. to be described. Given the rapid discovery of novel species, an increase in the number of species of regulatory

concern, and the prevalence of encountering *Phytophthora* spp. in diagnostic labs, a contemporary tool for identification of *Phytophthora* isolates to a species level is urgently needed.

A tool for rapid and easy identification of *Phytophthora* spp. would have to fulfill several requirements. Ideally, this tool should be sequence based because classical morphology is unable to distinguish many of the species currently recognized. For example *P. infestans*, *P. ipomoeae*, and *P. mirabilis* cannot easily be distinguished based on morphology alone because these species are essentially identical for sporangia (semipapillate and caducous) and oospore morphology (7,8,12). Yet, analysis of several sequences clearly distinguishes these species (8). A contemporary *Phytophthora* spp. identification tool also should be robust enough so that most laboratories with a thermocycler, basic molecular capability, and web access could make use of it.

Traditionally, molecular identification of *Phytophthora* spp. is accomplished by polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region followed by either restriction analysis (5) or direct sequencing and a BLAST search against GenBank or other databases. However, molecular identification based on the ITS region is not without its pitfalls (23). Although PCR-restricted fragment length polymorphism (RFLP) analysis of the ITS region can identify many species, currently, data for only one-half of the described species are available, and there are examples where ITS PCR-RFLP is not effective for differentiating closely related species (e.g., clade 1c species such as *P. infestans* and *P. mirabilis*). Identification can also be a challenge when using BLAST analysis with ITS sequences because there can be minimal or no differences between some species (e.g., clade 1c species) or, in some cases, intraspecific variation can blur the boundaries between species (e.g., *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* have identical ITS sequences). Due to its historical importance for the identification and phylogenetic analysis of the genus and the large number of sequences that have been deposited in GenBank, the ITS region remains an important locus for molecular identification; however, additional regions also have

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been examined (2,24,28,32). As more sequence data is collected from a wider range of geographically diverse isolates, the utility of alternative loci for accurate species identification will increase.

Perhaps the most important component for accurate molecular identification to the species level is the underlying database that is used. BLAST searches can be challenging once databases accumulate sequences that are mislabeled, of poor quality, or otherwise problematic (23). Thus, good reference libraries for identification of microorganisms need to be curated for quality. As of 26 March 2009, GenBank contained 47,794 *Phytophthora* DNA sequences, of which 2,741 were for the ITS region. Certain species are deeply represented, with over 100 ITS sequences submitted: *P. capsici* ($n = 199$), *P. cactorum* ($n = 176$), *P. cinnamomi* ($n = 166$), *P. nicotianae* ($n = 163$), *P. cryptogea* ($n = 152$), *P. infestans* ($n = 146$), and *P. citricola* ($n = 146$). Some of the species counts are inflated because several species look alike, such as those in the *P. citricola* complex that have recently been split into several species, including *P. plurivora*, *P. multivora*, and *P. citricola* (21). However, most species are represented only once and many sequences are from undescribed or poorly characterized species. GenBank is not actively curated in regards to correct identification of isolates associated with submitted sequences. Thus, problems exist for sequences deposited of incorrectly identified species. There is a need for a reliable, curated database for *Phytophthora* spp. identification.

Recently, a *Phytophthora* database (accessible at <http://www.phytophthoradb.org/>) was established to provide a tool for *Phytophthora* spp. identification and monitoring (30). This database is a valuable resource for *Phytophthora* biologists because it includes (as of 19 October 2010) over 4,650 sequences for upward of 2,400 isolates belonging to 106 *Phytophthora* spp. Currently, the database includes data for nine nuclear sequence loci, including the ITS region and the mitochondrially encoded cytochrome oxidase 2 gene (*cox2*) and the spacer region between *cox1* and *cox2*. This database houses information for populations of different *Phytoph-*

thora spp. and provides tools for phylogenetic tree building, generation of virtual restriction patterns for species of interest, and BLAST analysis for species identification.

The nuclear ribosomal RNA (rRNA) genes (small subunit, large subunit, and 5.8S) are organized in a cluster but separated by two ITS regions (ITS1 and ITS2; Fig. 1A). Typically, in eukaryotic genomes, the rRNA genes are repeated in tandem arrays in the order of several hundred or, possibly, thousands of copies (20). The spacer regions ITS1 and ITS2 that are transcribed but not translated are more variable than the actual rRNA genes. The spacer regions diverge rapidly enough to be informative in distinguishing most (but not all) *Phytophthora* spp. (4).

The cytochrome c oxidase subunit 1 and 2 genes (*cox1* and *cox2*, respectively) are present as a gene cluster on the mitochondrial chromosome in *Phytophthora* (as well as in *Pythium* and many plant species; 27) (Fig. 1B). Due to interspecific sequence variation, the spacer region between these two genes is useful for development of species-specific markers (29,34) as well as for sequence-based identification of many species (F. Martin, unpublished). One advantage of using this region for species identification is that it can be directly amplified from infected plant tissue as part of the *Phytophthora* genus-specific amplicon commonly used in a conventional PCR diagnostic assay for this genus (23), thereby eliminating the need for culturing and DNA extraction before BLAST analysis.

With creation of www.Phytophthora-ID.org, our objective was to provide a simple yet robust site that is easy to use and based on both the ITS and *cox* spacer regions. The *cox* spacer region was included because this locus encompasses a genus-specific amplicon used in molecular diagnostics for *Phytophthora* (23) and exhibits suitable interspecific variation to be useful for development of species-specific markers. We developed a database of sequences that is regularly updated to be selective for sequence accessions that come from trusted sources. We also validated the database

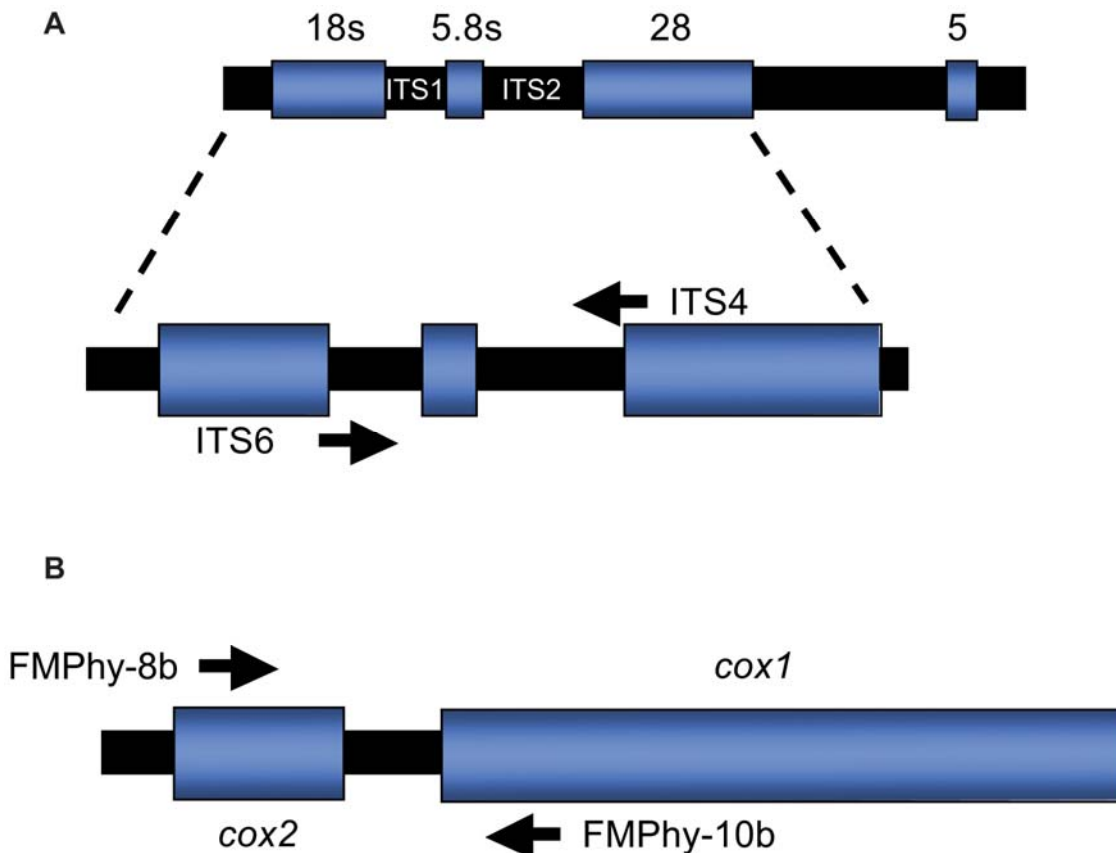


Fig. 1. Diagrammatic representation of the **A**, internal transcribed spacer (ITS) and **B**, cytochrome oxidase (*cox*) spacer regions in *Phytophthora* spp. The ITS region is polymerase chain reaction amplified using primers ITS4 and ITS6 whereas the *cox* spacer region is amplified using primers FMPhy-8b and FMPhy-10b.

using an extensive sample of *Phytophthora* spp. The purpose of this article is to report on progress in the development of Phytophthora-ID and the validation of its data sets using an extensive sample of *Phytophthora* spp. It is expected that Phytophthora-ID will also help in development and maintenance of culture collections (22).

Materials and Methods

Phytophthora-ID content and implementation. Phytophthora-ID provides a web-based platform for sequence-based BLAST searches against two regularly updated sequence databases. The two databases available in FASTA format (see <http://www.ncbi.nlm.nih.gov/BLAST/fasta.shtml>) include the ITS region sequences and the *cox1* and -2 spacer region. The web deployment of the database was accomplished using the Personal Sequence Database (14).

Both data sets presented on the Phytophthora-ID website list only sequences that are associated with (i) a published *Phytophthora* spp. description; (ii) the *Phytophthora* phylogenetics references by Cooke et al. (4), Martin and Tooley (28), and Blair et al. (2); or (iii) in the case of the *cox* spacer region, from isolates resequenced for this effort (F. Martin, *unpublished*). Further, novel taxa tentatively named are selectively included and labeled as *P.* taxon "X"; as in, for example, *P.* taxon "asparagi". Some *cox* spacer sequences are currently unpublished and in preparation for publication. The complete list of ITS sequences, *cox* spacer sequences, GenBank accession numbers, and *Phytophthora* spp. included can be downloaded at www.Phytophthora-ID.org or as supplements (Supplementary Tables 1 and 2).

Species for which several nonidentical sequences exist are also included in the database. However, identical sequences for different isolates of the same species are excluded to avoid redundancy. For some species, neither ITS sequences nor voucher specimens were available; these species were generally described before the molecular era and, thus, could not be included. The broad species diversity sampled, including all known ITS sequences for *Phytophthora* spp. described to date, is shown in Figure 2.

PCR reactions and sequencing. PCR reactions for the ITS or *cox* spacer regions were conducted using either purified DNA or directly from a mycelial tuft lifted off a colony. For the ITS sequencing, genomic DNA was extracted from mycelia using the FastDNA SPIN kit (MP Biomedicals, LLC, Solon, OH) (18) whereas, for the *cox* spacer region, DNA was isolated as previously described (1). Mycelial tufts were obtained by scraping along the surface of a plate (1 to 2 cm) toward the margins of a growing culture where sporangia were present and lifting hyphae using a sterile, dry toothpick. The mycelial tuft was then placed into a 100- μ l PCR tube containing distilled water and heated to 95.9°C for 5 min. PCR reactions were conducted on ABI 9700 or Verity thermocyclers (Applied Biosystems Inc., Foster City, CA) under the PCR conditions and protocols presented below. Amplicons were stored at -20°C until sequenced. PCR amplicons of the ITS region were sequenced in forward and reverse at the Center for Genome Research and Biocomputing (Oregon State University, Corvallis) on an ABI 3730 capillary sequencer using standard reagents (Applied Biosystems Inc.). The *cox* spacer sequences were generated in both directions at the Pennsylvania State

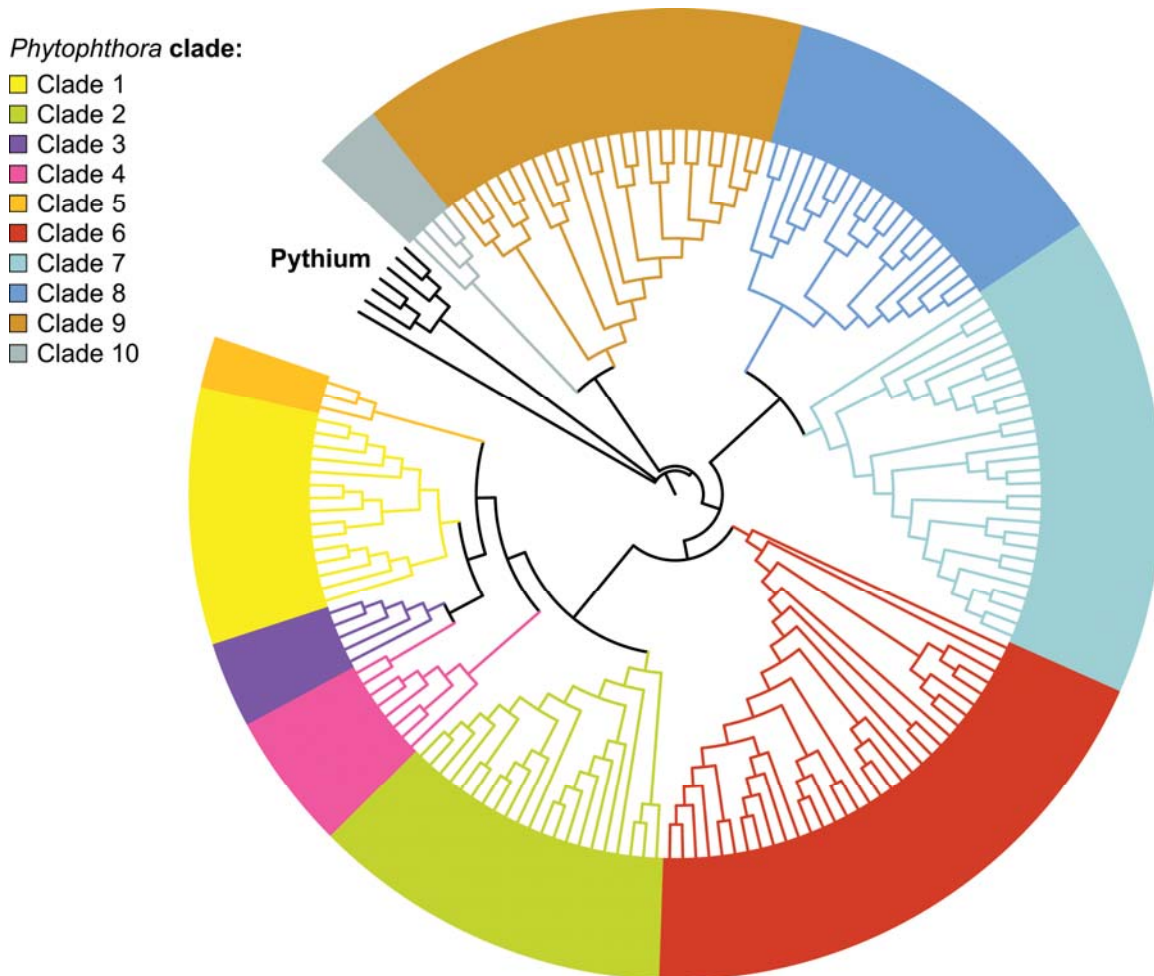


Fig. 2. Dendrogram showing the full clade diversity included in Phytophthora-ID. Dendrogram was constructed using all internal transcribed spacer (ITS) sequences contained in Phytophthora-ID after automated alignment using Clustal-W (33) followed by neighbor joining and rooting the tree with several *Pythium* outgroups. The tree was created using the Interactive Tree of Life (25). Note that this tree only depicts diversity and is not meant to replace a proper phylogenetic analysis as presented, for example, in Blair et al. (2).

the *Phytophthora* database project in the course of sequencing the *cox2* gene for phylogenetic analysis of the genus (F. Martin and M. D. Coffey, unpublished). The majority of the *cox2* gene, the spacer region, and the 5' end of the *cox1* gene were amplified using primers FM35 and Phy-10b, with nested primers FM79 and FM80 used for sequencing the spacer region and flanking coding regions (26,28,29). For amplification of the genus-specific amplicon from either plant samples or purified pathogen DNA, the primers FMPhy-8b and FMPhy-10b were used (29; Fig. 1B). Amplification reactions were conducted in final concentrations of genomic DNA at 1 to 3 ng μl^{-1} , 200 μM dNTP (2.0 mM), 0.4 μM ITS6 Phy-8b and Phy-10b primers, 2% glycerol, 3 mM MgCl_2 , 1 \times buffer, and AmpliTaq polymerase (Applied Biosystems) at 0.05 unit/ μl . PCR conditions were as follows: 1 cycle of denaturation at 95°C for 3 min; 35 cycles of 1 min at 95°C, 1 min of annealing at 65.5°C, and 1 min of extension at 72°C; followed by 1 extension cycle at 72°C for 5 min. *Phytophthora* spp. typically yield PCR products in the range of 450 bp.

When doing amplifications from infected plant tissue, the concentration of pathogen DNA relative to plant DNA can be very low, and two rounds of amplification may be necessary to get enough template to clearly visualize on a gel (23). The first-round amplification can be diluted 1:100 and the amplification repeated without the addition of glycerol to the master mix and using a 64°C annealing temperature. Additional details on amplification and sequencing of this region from infected plant tissue can be found in the molecular diagnostics section of the *Phytophthora* Database (www.phytophthoradb.org).

Application of Phytophthora-ID to nursery samples. Phytophthora-ID was validated using an extensive *Phytophthora* sampling effort in commercial nurseries. *Phytophthora* cultures used to evaluate the database were maintained on cleared 10% V8 agar medium (100 ml of V8 juice, 2 g of CaCO_3 , 30 mg of β -sitosterol, 15 g of agar, and 900 ml of deionized water) in a 20°C incubator in the dark as described previously. We used Phytophthora-ID to identify *Phytophthora* spp. collected in nursery environments during 2006 to 2009 based on ITS sequencing. Species were isolated from different nursery crops, mostly *Rhododendron*, *Viburnum*, *Pieris*, and *Kalmia* spp., but also potting mix, soil, and irrigation and pond water. In total, 700 isolates were collected from four commercial ornamental nurseries in Oregon.

Use of database and interpretation of search results. To use Phytophthora-ID, sequences for a given isolate in FASTA format are submitted for BLAST searching at either the ITS or *cox* spacer BLAST window (Fig. 3A). Users can specify whether they want to have plain-text output or graphical output as well as the number of top hits (Fig. 3B). After submission, Phytophthora-ID returns a BLAST result window (Fig. 3C).

DNA-based *Phytophthora* spp. identification is an iterative process that, at times, requires further work and might include consideration of morphological traits. Interpretation of sequence reads and BLAST results requires experience. Generally, the best match shown at the top is the best estimate of species identity, although care has to be taken when interpreting these results. The scores derived from BLAST analysis can be influenced by length of coverage as well as sequence identity; therefore, it is important to look for nearly complete query coverage with very high sequence identity and a very low E value. Generally, when sequences are 100% identical, then the *Phytophthora* sp. has been identified. If identity is <100% but $\geq 99\%$, a putative match was made, and it is suggested that follow-up identification of key morphological characters using microscopy be conducted. If identity is <99%, a new species might be detected and additional work will be required. In this case, sequencing of the *cox* spacer region and BLAST search in Phytophthora-ID is suggested, followed by sequencing of additional loci and searching of PhytophthoraDB (30). It is important to recognize that one locus is not always suitable for identification of all species; low levels of sequence divergence may be observed among phylogenetically closely related species and differentiation to a species level may be further complicated due to

intraspecific variation. Therefore, use of multiple loci will often improve the accuracy of identification.

Drawing conclusions from a BLAST analysis can be challenging for some species because several scores can be obtained for the same species or multiple species can be encountered with the same score, thus complicating species identification. A good example of this was found with some closely related clade 8 species in a recent evaluation of ITS (over 300 isolates) and *cox2* gene sequences. Although the ITS data could clearly distinguish *P. drechsleri* from *P. cryptogea*, it could not differentiate among *P. cryptogea*, *P. erythroseptica*, and *P. richardiae*. This was due to intraspecific variation among these closely related species providing an overlap in sequence identity scores among species. Conversely, the *cox2* gene data could separate all these species, highlighting the importance of using multiple loci for final isolate identification. Therefore, using multiple loci can often improve the accuracy of identification. Another approach for reducing the impact of intraspecific variation on species identification is selectively using only those portions of the ITS region that are highly conserved on an intraspecific level in the BLAST analysis, thereby eliminating the potential for obtaining overlapping scores (6).

Validation of database. During validation of Phytophthora-ID, *Phytophthora* isolates collected from four commercial nurseries could be unambiguously identified to species where species were known. Nurseries were dominated by a few species, including *P. plurivora*, *P. cinnamomi*, *P. syringae*, the poorly characterized species complex *P. sp. PgChlamydo*, *P. citrophthora*, and *P. cryptogea* (Fig. 4). *P. gonapodyides* was routinely retrieved from irrigation water.

Conclusions

Phytophthora-ID is a user-friendly, simple, and robust tool for identification of *Phytophthora* spp. Phytophthora-ID is complementary to the *Phytophthora* database (30) and, in fact, used carefully selected data for the *cox* spacer and ITS regions that we selectively resequenced from that resource to populate the databases. The unique contribution of Phytophthora-ID is that the underlying sequence databases used for BLAST searches were carefully assembled from trusted sources and will continue to be curated to ensure accuracy.

Some similar databases are in existence. For example, Unite provides a resource for identification of fungi (1). Fusarium-ID provides a resource for *Fusarium* spp. identification (13). PhytophthoraDB provides a parallel resource to Phytophthora-ID housing sequence data at both the intra- and interspecific level for *Phytophthora* spp. (30). Sequences for new species can be added to Phytophthora-ID upon request to the authors, who will decide if sequences will be deposited. Generally, only newly described species accompanied by a peer-reviewed publication or tentative,

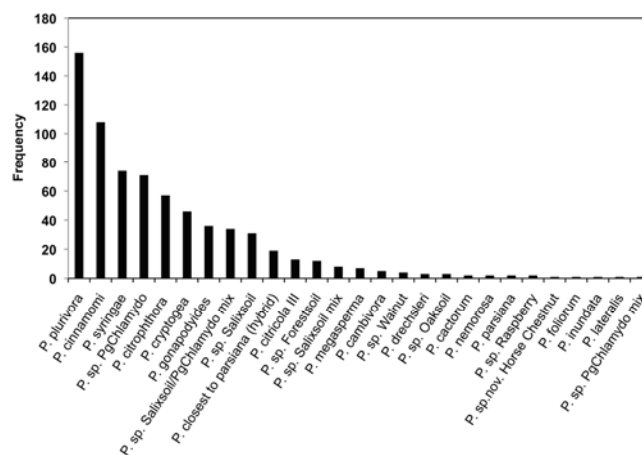


Fig. 4. Frequency of 700 *Phytophthora* spp. identified during a survey of four commercial Oregon nurseries conducted during 2006 to 2009.

novel taxa (labeled temporarily as *P. taxon* “X” until published) will be added to this resource. There also exist databases at the intraspecific level housing genotypic information for certain species such as *P. infestans* (9) and *P. ramorum* (15,16).

Phytophthora-ID is an ongoing project that the authors will continue to update regularly. Both the ITS and *cox* spacer databases can be downloaded. Sequences for newly discovered species will be included as the literature expands. Authors are encouraged to submit sequences, DNA, or cultures for novel species to the lead author.

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