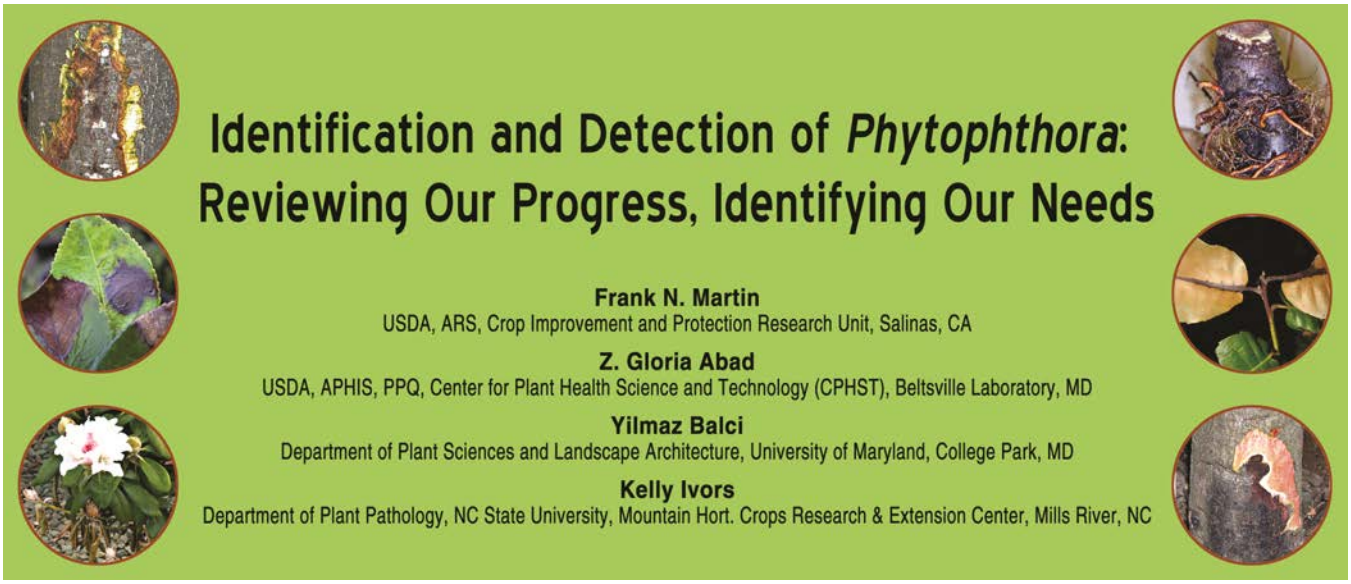

With the increased attention given to the genus *Phytophthora* in the last decade in response to the ecological and economic impact of several invasive species (such as *P. ramorum*, *P. kernoviae*, and *P. alni*), there has been a significant increase in the number of described species. In part, this is due to the extensive surveys in historically underexplored ecosystems (e.g., forest and stream ecosystems) undertaken to determine the spread of invasive species and the involvement of *Phytophthora* species in forest decline worldwide (e.g., oak decline). Brasier (41) cited the number of described species in 1999 at approximately 55, and there has been an increase of an additional 50 species or distinct taxonomic entities described between 2000 and 2007. This represents a near doubling in eight years! Érsek and Ribeiro (80) recently updated the list of described species to 100; since then, additional species have been named (some provisional), raising the total to 117 (Table 1) with a number of other distinct taxonomic entities in the process of formal description. The number of species will likely continue to increase as more surveys are completed and greater attention is devoted to clarifying phylogenetic relationships and delineating boundaries in species complexes. The development of molecular resources, including the recent comprehensive multigene phylogenetic analysis of the genus (32), the availability of credible sequence databases to simplify identification of new species (www.phytophthoradb.org, www.phytophthora-id.org, www.q-bank.eu, and www.boldsystems.org), and the sequencing of several genomes (107,278) have provided a solid framework to move forward. Gaining a better understanding of the biology, diversity, and taxonomic relationships within the genus will be important for the improvement of identification and diagnostic protocols. This information is much needed considering the impact invasive or exotic *Phytophthora* species have had on natural ecosystems and the regulatory issues associated with their management. Reviews by Cooke et al. (61) and O'Brien et al. (217) are noteworthy for providing additional information on molecular identification and detection.

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Table 1 is also available, in a slightly different format, online.

http://dx.doi.org/10.1094/PDIS-12-11-1036-FE

Taxonomy of the Genus *Phytophthora*

Numerous studies on the taxonomy of *Phytophthora* have been published since the initial description of the type species of the genus, *Phytophthora infestans* (Mont.) de Bary, in 1876 (reviewed in Erwin and Ribeiro, 82). Most of the publications contain dichotomous keys for species identification with the exception of Stamps et al. (253), which includes a tabular format based on keys developed by Waterhouse (286,287). Waterhouse (286) introduced the concept of morphological groupings (I-VI) "with the intent to solely serve as an aid to species identification, and not meant to imply a natural classification". Newhook et al. (213) and Stamps et al. (253) also included these groupings in their taxonomic evaluation of the genus. More recent attempts to simplify identification of species include a manual for identification of 60 species of *Phytophthora* by integration of a dichotomous key with a DNA fingerprinting technique based on polymerase chain reaction (PCR)-single strand conformational polymorphism (SSCP) (88) and a Lucid Key for identification of 55 common *Phytophthora* spp. based on a series of interactive computer matrices (230). Cline et al. (55) have published an online list of *Phytophthora* spp. occurring in the United States as well as species occurring elsewhere with a hyperlink for each species to the USDA SMML database that includes host range, distribution, and supporting literature. Recently, Kroon et al. (166) provided an update on the taxonomy of the genus.

Phylogeny of the Genus *Phytophthora*

Historically, the genus *Phytophthora* has been placed in the Pythiales with *Pythium* and related genera, but more recent phylogenetic analysis with the large (LSU) or small (SSU) ribosomal DNA (rDNA) sequences or *cox2* gene has indicated a closer affiliation with downy mildews and white rusts (*Albugo* spp.) in the Peronosporales (21,261). However, additional multigene analysis with a larger number of downy mildew species is needed to better characterize this relationship and the placement of *Phytophthora* spp. in clades 9 and 10 (32). The relationship between the Peronosporales and *Pythium* (Pythiales) needs clarification as well. Recently, a new genus, *Phytopythium,* was erected to accommodate an inconsistency between taxonomic and phylogenetic grouping for these two genera (17), and it is likely that additional taxonomic revisions of the Peronosporomycetidae with additional genera added to the analysis will be needed to fully resolve other taxonomic conflicts.

A comprehensive multilocus phylogenetic analysis of 82 species in the genus was recently completed by Blair et al. (32), and for the most part the phylogenetic groupings were similar to those ob-

served in the internal transcribed spacer (ITS) analysis by Cooke et al. (58) and the multilocus analysis of Kroon et al. (165). While Cooke et al. (58) reported 8 major clades, with the addition of more recently described species Blair et al. (32) observed 2 additional groupings (clades 9 and 10) that were basal to the prior 8 clades. The same isolates used in Blair et al. (32), along with more recently described species, were also used in a current mitochondrial multilocus analysis, and with a few exceptions, results were similar (Fig. 1). While phylogenetic groupings have exhibited some similarity with the morphological groupings of Waterhouse (286) and placement of species on individual clades, this was not an absolute correlation, and a number of exceptions were observed. There was no consistent correlation between reproductive strategy (homothallic versus heterothallic) or antheridial attachment (paragynous versus amphigynous) with phylogenetic grouping. A variety of other nuclear and mitochondrial loci (discussed below) have been used in phylogenetic analysis of the genus (165,195, 196,283; see also www.phytophthoradb.org).

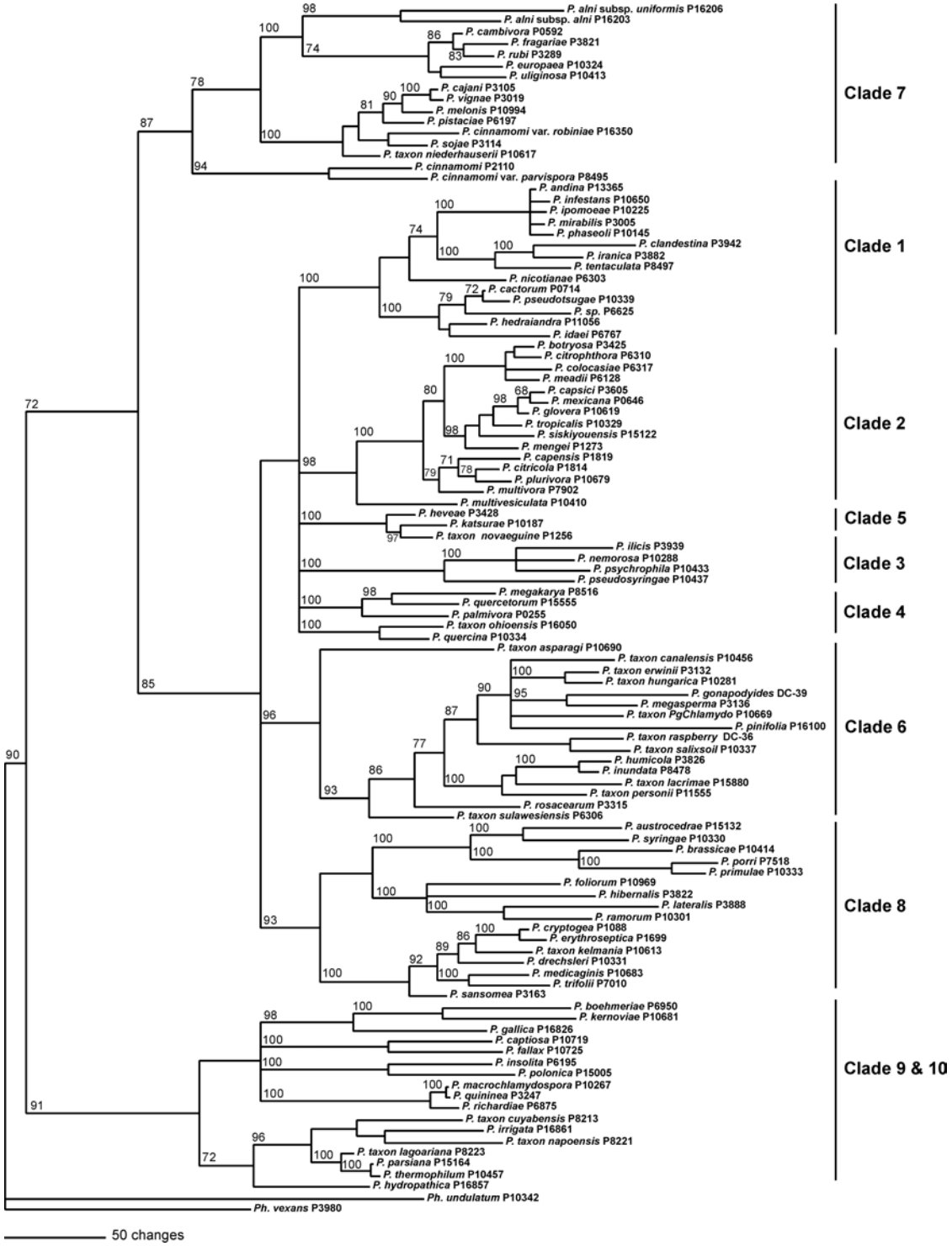

Fig. 1. Maximum parsimony analysis of concatenated *cox2, nad9, rps10,* and *secY* genes from a range of *Phytophthora* spp. with *Phytopythium vexans* and *Ph. undulatum* as outgroups (F. Martin, J. Blair, and M. Coffey, *unpublished*). The numbers at the nodes represent bootstrap values (below 70% is not presented) with the clade labeling the same as Blair et al. (32).

Table 1. List of described *Phytophthora* species and their asexual and sexual morphological features, temperature growth range, Waterhouse morphological grouping, and placement in phylogenetic analysis

Species	Asexual phase							Sexual phase				Temperature			
	PA[a]	C[b]	PE[c]	PR[d]	SPO[e]	CH[f]	HS[g]	H[h]	OOG[i]	AN[j]	OOS[k]	OPM[l]	MAX[m]	WG[n]	Clade[o]
P. cactorum	P	C	S	-	CLS	CH/-	-	HO	S	P	P/A	21-27	<30C	I	1a
P. hedraiandra	P	C	S	-	SS/UN	-	HS/-	HO	S	P/A	SA	24-27	<30C	I	1a
P. idaei[p]	P	-	-	-	SS	-	-	HO	S	P/A	A	18-24	<27C	I	1a
P. pseudotsugae	P	-	-	-	SS	-	HS	HO	S	P/A	P/A	18-24	<27C	I	1a
P. clandestina[p]	P	C/-	S	-	UN	-	HS	HO	S	P/A	P/A	18-22	<28C	I	1b
P. iranica	P	-	-	-	CS	-	HS	HO	S	P/A	A	24-30	<33C	I	1b
P. tentaculata[p]	P	C	S	-	UN/SS	CH	HS	HO	S	P/A	P/A	21-24	<30C	I	1b
P. nicotianae	P	C/-	S	-	SS	CH	HS	HT	S	A	P/A	24-33	>35C	II	1
P. andina	SP	C	S	-	CS	-	-	HT	S	A	P/A	20-24	<30C	IV	1c
P. infestans	SP	C	S	-	CS	-	-	HT	S	A	P/A	18-20	<24C	IV	1c
P. ipomoea	SP	C	S	-	CS	-	-	HO	S	A	P/A	18-24	<24C	III	1c
P. mirabilis	SP	C	S	-	CS	-	-	HT	S	A	P/A	18-20	<24C	IV	1c
P. phaseoli	SP	C	S/M	-	SS	-	-	HO	S	A	A	18-24	<27C	III	1c
P. botryosa[p]	P	C	M	-	SS	CH/-	-	HT	S	A	P	21-26	<32C	II	2a
P. himansilva	P	C/-	M	-	SS/UN	-	-	HO	S	P/A	A	20-25	<30C	II	2a
P. meadii[p]	P/SP	C	M	-	SS/UN	CH/-	-	HT/HO	S	A	SA	25-30	<33C	II	2a
P. citrophthora	SP	-	-	-	IB	CH/-	-	HT	S	A	P	24-30	<33C	IV	2a
P. colocasiae	SP	C	M	-	IB	CH	HS	HT	S	A	A	21-27	<33C	IV	2a
P. inflata	SP	-	-	-	UN	-	HS/-	HO	S	P	P	25-30	<35C	III	2a
P. capsici	P	C	L	-	SS	-	HS	HT	S	A	P	27-33	>35C	II	2b
P. glovera	P	-	-	-	SS	-	-	HO	S	P/A	A	20-25	<30C	I	2b
P. mexicana	P	-	-	-	UN/SS	CH	-	HT	S	A	P	25-30	<35C	II	2b
P. tropicalis	P	C	L	-	UN/SS/U	CH	-	HT	S	A	P	27-30	<33C	II	2b
P. siskiyouensis	SP	C/-	L	-	UN/SS	-	HS	HO	S/tb	P/A	P/A	21-25	<27C	III	2b
P. frigida[p]	P	C	S	-	SS	CH	HS	HT	S	A	A	20-25	<30C	II	2
P. bisheria	SP	-	-	-	UN	-	HS	HO	S	P	A	18-24	<30C	III	2
P. capensis	SP	-	-	-	UN	-	-	HO	S	P	P	18-24	<28C	III	2
P. citricola	SP	-	-	-	SS	-	-	HO	S	P	P	24-30	<31C	III	2
P. elongata	SP	-	-	-	UN/SS	-	-	HO	S/tb	P	P/A	20-25	<30C	III	2
P. mengei	SP	-	-	-	SS/UN	-	-	HO	S/tb	P	P/A	20-25	<33C	III	2
P. multivesiculata[p]	SP/NP	-	-	PR	UN/SS	-	HS	HO	S/tb	P/A	A	21-27	<35C	III	2
P. multivora	SP	-	-	-	SS	-	HS	HO	S/tb	P	P	18-30	<30C	III	2

(*continued on next page*)

[a] Sporangia papillae with P = papillate (≥3.5 µm), SP = semipapillate (≤3.5 µm), NP = nonpapilate.
[b] Caducity of sporangia with C = caducous, Cl- = sometimes caducous, and "-" = not caducous.
[c] Pedicle length with S = short (<5 µm), M = medium (5-20 µm), L = long (>20 µm), and "-" = no pedicle.
[d] Proliferating sporangia = PR, "-" = nonproliferating.
[e] Sporangiophore form CLS = Close sympodium, SS = simple sympodial, un = unbranched, CS = compound sympodial, IB = irregularly branched, U = umbellate, D.DM = Downy mildew shape, and ? = no reported.
[f] Formation of chlamydospores with CH = formed, CH/- = sometimes formed, and "-" = not formed.
[g] Formation of hyphal swellings with HS = formed, HS/- = sometimes formed, and "-" = not formed.
[h] Reproductive strategy with HO = homothallic and HT = heterothallic.
[i] Oogonial wall with S = smooth, O = ornamented, S/O = smooth and ornamented, S/SW = smooth and slight wavy wall, O/tb = ornamented wall and tapered base, S/tb = smooth wall and tapered base.
[j] Antheridial attachment with P = paragynous, A = amphigynous, and P/A = both present.
[k] Space between oospore and oogonial wall with P = plerotic, A = aplerotic, P/A = both plerotic and aplerotic, and SA = slightly aplerotic.
[l] Optimum temperature for growth.
[m] Maximum temperature for growth.
[n] Morphological group of Waterhouse (286).
[o] Phylogenetic clade (32,58).
[p] Species that are on the list "Prioritization of *Phytophthora* of Concern to the United States" (245).
[q] ? refers to unknown character, species is sterile or does not produce sexual stage.

Table 1. (*continued from previous page*)

Species	Asexual phase							Sexual phase				Temperature			
	PA[a]	C[b]	PE[c]	PR[d]	SPO[e]	CH[f]	HS[g]	H[h]	OOG[i]	AN[j]	OOS[k]	OPM[l]	MAX[m]	WG[n]	Clade[o]
P. pini	SP	-	-	-	SS	-	HS/-	HO	S	P	P	18-27	<31C	III	2
P. plurivora	SP	-	-	PR	SS	-	-	HO	S/tb	P	P	18-27	<30C	III	2
P. ilicis	SP	C	S/M	-	SS	CH/-	-	HO	S	A	P	15-18	<24C	III	3
P. nemorosa	SP	C	M	-	SS	-	HS	HO	S	A	P/A	15-20	<21C	III	3
P. pseudosyringae	SP	C/-	S/M	-	SS	-	HS	HO	S/tb	P/A	P/A	21-24	<25	III	3
P. psychrophila	SP	C/-	M	-	SS	CH	-	HO	S/tb	A	P/A	15-21	<21C	III	3
P. alticola[p]	P	C	S	-	SS/CS	CH/-	HS	HO	S	P/A	A	25-30	<30C	I	4
P. arenaria	P	-	-	-	UN.gs	-	HS	HO	SW/tb	P	A	25-30	<32C	I	4
P. litchii	P	-	-	PR	D.DM	-	-	HO	S	P/A	P/A	ND	ND	I	4
P. palmivora	P	C	S	-	SS	CH	HS	HT	S	A	P/SA	24-30	<33C	II	4
P. quercetorum	P	-	-	-	UN/SS	CH/-	-	HO	S/tb	P	A	18-24	<30C	I	4
P. megakarya[p]	P	C	M	-	SS	CH/-	-	HT	S/tb	A	P/A	21-27	<27C	II	4
P. quercina[p]	P	-	-	-	SS	CH/-	HS	HO	S/tb	P	P/A	21-24	<25	I	4
P. heveae	P	-	-	-	SS	-	-	HO	S/tb	A	A	21-27	<27C	I	5
P. katsurae[p]	P	-	-	-	IB	CH/-	-	HO	O	A	SA	21-27	<32C	I	5
P. inundata	NP	-	-	PR	UN/SS	-	HS	HT	S	A	P/A	27-30	>36C	VI	6
P. gemini	NP			-	UN	-	HS	S	-	-	-	24-27	<33C	VI?	6
P. humicola	NP	-	-	PR	UN	-	HS	HO	S	P/A	SA	27-30	>36C	V	6
P. rosacearum	NP	-	-	-	UN	-	HS/-	HO	S	P	P/A	27-33	<33C	V	6
P. pinifolia[p]	NP	C/-	-/M	-	SS	-	HS/-	S	-	-	-	13-18	<20	VI?	6
P. thermophila	NP	-	-	PR	UN	CH	HS	S/HO	S	P	A	25-32	>35C	V?	6
P. litoralis	NP	-	-	PR	UN	CH/-	HS	S	-	-	-	25-30	<32C	VI?	6
P. fluvialis	NP	-	-	PR	UN	-	HS	S	-	-	-	28-32	>35C	VI?	6
P. gonapodyides	NP	-	-	PR	UN	-	HS	S/HT	S	A	P/A	24-27	<30C	VI?	6
P. megasperma	NP	-	-	PR	UN/SS	-	HS	HO	S	P	P/A	18-27	<27C	V	6
P. gibbosa	NP/SP	-	-	PR	UN	-	HS/-	HO	S/SW	A	A	25-30	<32C	V	6
P. gregata	NP	-	-	PR	UN	-	HS	HO	S/tb	P/A	A	20-25	<32C	V	6
P. alni* sub sp. *alni[p]	NP	-	-	PR	UN	-	-	HO	O/tb	A	P	18-24	<29C	V	7a
P. alni* sub sp. *multiformis[p]	NP	-	-	PR	UN	-	-	HO	O/S	P/A	P	21-24	<30C	V	7a
P. alni* sub sp. *uniformis[p]	NP	-	-	PR	UN	-	-	HO	S/O	A	P	24-27	<30	V	7a
P. cambivora	NP	-	-	PR	UN	-	HS	HT	O/tb	A	P	24-30	<32C	VI	7a
P. europaea	NP	-	-	PR	UN	-	HS/-	HO	S	P	A	21-27	<27C	VI	7a
P. fragariae	NP	-	-	PR	UN	-	HS	HO	S/tb	P/A	P/A	18-24	<27C	V	7a
P. rubi	NP	-	-	PR	UN/SS	-	-	HO	S/tb	A	P/A	15-21	<27C	V	7a
P. uliginosa	NP	-	-	PR	UN	-	HS	HO	S/tb	P/A	P/A	15-21	<21C	V	7a
P. cajani[p]	NP	-	-	PR	UN	-	-	HO	S	A	P	28-32	<33C	VI	7b
P. cinnamomi	NP	-	-	PR	UN	CH	HS	HT	S	A	P	27-30	<30C	VI	7b
P. melonis[p]	NP	-	-	PR	UN	-	HS/-	HT	S	A	P/A	25-28	<35C	VI	7b
P. pistaciae[p]	NP	-	-	PR	UN	-	HS	HO	S	P/A	A	25-30	<33C	V	7b
P. sojae	NP	-	-	PR	UN/SS	-	HS	HO	S/tb	P/A	P/A	23-30	<33C	V	7b
P. vignae[p]	NP	-	-	PR	SS	CH	HS	HO	S	A	A	28-30	>35C	V	7b
P. cinnamomi* var. *parvispora[p]	NP	-	-	PR	SS	CH	HS	HT	S	A	P	?	<30C	VI	7b
P. cryptogea	NP	-	-	PR	UN	-	HS	HT	S	A	P	24-30	<30C	VI	8a
P. drechsleri	NP	-	-	PR	UN/SS	-	HS	HT	S	A	P/A	27-33	>35C	VI	8a
P. erythroseptica	NP	-	-	PR	SS	-	HS	HO	S	A	A	21-30	<30C	V	8a
P. medicaginis	NP	-	-	PR	UN/SS	CH	HS	HO	S	P/A	P/A	27-33	<33C	V	8a

(*continued on next page*)

Table 1. (*continued from previous page*)

Species	Asexual phase							Sexual phase				Temperature			
	PA[a]	C[b]	PE[c]	PR[d]	SPO[e]	CH[f]	HS[g]	H[h]	OOG[i]	AN[j]	OOS[k]	OPM[l]	MAX[m]	WG[n]	Clade[o]
P. sansomeana	NP	-	-	PR	UN	-	HS	HO	S	P/A	P/A	24-30	<33C	V	8a
P. trifolii	NP	-	-	PR	UN/SS	-	HS	HO	S	P/A	A	21-27	<30C	V	8a
P. brassicae	SP	C	S	-	SS/U	-	HS	HO	S	P/A	A	15-21	<25C	III	8b
P. porri[p]	SP	-	-	-	SS	-	HS	HO	S	P/A	A	15-18	<20	III	8b
P. primulae[p]	NP	-	-	-	UN	-	HS	HO	S	P/A	A	15-21	<27C	V	8b
P. foliorum	NP/SP	C/-	-/S	PR	SS	CH/-	-	HO	S	P	P	18-24	<30C	V	8c
P. hibernalis	SP	C	L	-	UN/SS/U	-	-	HO	S	P/A	P/A	15-21	<25C	III	8c
P. lateralis	NP	-	-	PR	SS/UN	CH	-	S/HO	S	P	P	15-24	<26C	V	8c
P. ramorum	SP	C	S	-	SS	CH	-	HT	S	A	P	15-21	<27C	IV	8c
P. austrocedrae[p]	SP	-	-	-	UN	-	HS	HO	S	A	A	15-21	<22C	III	8d
P. obscura	SP	-	-	-	UN	-	HS	HO	S	P	P/A	20-25	<27C	III	8d
P. syringae	SP	-	-	-	SS	-	HS	HO	S	P/A	P/A	18-24	<24C	III	8d
P. aquimorbida	NP	-	-	PR	UN	CH	HS	HO	S/tb	A rare	P	25-30	<39	V	9
P. irrigata	NP	-	-	PR	UN	-	-	HT	S	A	P	25-30	<30C	VI	9
P. hydropathica	NP	-	-	PR	UN	CH	HS	HT	S	P/A	P	25-30	<37	VI	9
P. parsiana	NP	-	-	PR	UN	-	HS	HT	S	A	A	25-30	<35C	VI	9
P. chrysanthemi	NP	-	-	PR	?	CH	HS	HO	S	P/A	A	24-30	<35C	V	9
P. polonica	NP	-	-	PR	UN	CH	HS	HO	S	P/A	P/A	24-27	<33C	V	9
P. insolita	NP	-	-	PR	UN	-	HS	S/HO	S?	-	P?	33-36	<36C	V	9
P. captiosa[p]	NP	-	-	PR	UN?	-	-	HO	S	A	P/A	18-24	<27C	V	9
P. fallax	NP	-	-	PR	SS	CH/-	-	HO	S	P/A	P/A	15-20	<25C	V	9
P. constricta	NP	-	-	PR	UN.gs	-	-	HO	S/tb	P	P/A	20-22	<32C	V	9
P. richardiae	NP	-	-	PR	UN	-	HS	HO	S	P/A	P	20-24	<31C	V	9
P. macrochlamydospora[p]	NP/SP	-	-	PR	UN	CH	HS	S	-	-	-	23-30	<36C	VI?	9
P. quininea[p]	NP	-	-	-	SS	-	HS	S	-	-	-	22-27	<30C	VI?	9
P. kernoviae[p]	P	C	M	-	SS/UN	-	-	HO	S/tb	P/A	P	18-20	<26C	I	10
P. morindae	P	C	M/L	-	U	-	-	HO	S/tb	P/A	P/A	21-27	<27C	I	10
P. boehmeriae[p]	P	C	S	-	UN	-	-	HO	S	A	P/A	21-27	<28C	I	10
P. gallica	NP	-	-	PR	UN	CH	HS	S	-	-	-	20-21	<24	VI?	10
No specimens available in culture collections															
P. japonica[p]	NP	-	-	PR	SS	-	HS	HT	S	P/A	A?	24-27	<30C	VI	ND
P. italica[p]	P	-	-	-	UN	CH/-	-	HO	S	P	A	22-26	<35C	I	ND
Have not been cultured, described based on morphological features observed in infected tissue.															
P. leersiae	ABSENT							HO	S	P	A	ND	ND	?	ND
P. oryzo-bladis	NP	-	-	PR	UN/SS	-	-	HO	S	A	P/A	ND	ND	V	ND
P. polygoni[p]	SP	C	M	-	UN/SS	CH/-	-	HO	S	P	A	ND	ND	III	ND
P. verrucosa	NP	-	-	-	SS	-	HS	HO	S	P/A	P/A	ND	ND	V	ND
P. cyperi	SP	-	-	-	SS	-	HS	HO	S	P/A	A	ND	ND	III	ND
P. cyperi-bulbosi	SP	C	S	-	SS	-	-	HO	O	P	P	ND	ND	III	ND
P. eriugena	P	-	-	-	SS	-	HS	HO	S	P/A	A	ND	ND	III	ND
P. lepironiae	SP	C	M	-	UN	-	-	HO	S	P	P	ND	ND	III	ND

Designed by Z. Gloria Abad USDA-APHI-CPHST

While the overall phylogeny of the genus has been clarified by multigene analysis, ambiguities still remain for some closely related groupings, and there is taxonomic uncertainty with some morphologically similar species complexes. In the last few years, clarification of the *P. megasperma* complex (43) and other clade 6 species have been the focus of several labs with species such as *P. fulvialis*, *P. gibbosa, P. gregata, P. litoralis*, *P. pinifolia, P. rosacearum,* and *P. thermophila* recently described (75,109,152,154). There are also a number of clade 6 provisional species awaiting further analysis and formal species description (*P. taxon* PgChlamydo, *P. taxon* raspberry, *P. taxon* salixsoil, *P. taxon* canalensis, *P. taxon* erwinii, *P. taxon* hungarica, *P. taxon* oregonsis, and *P. taxon* paludosa). Likewise, *P. cryptogea* and *P. drechsleri* have long been a challenge to differentiate based on morphological features alone (reviewed in Erwin and Ribeiro, 82), and while different groupings were observed based on isozyme (204) and ITS phylogenetic analysis (87), taxonomic clarity has been elusive. The multigene analysis of Mostowfizadeh-Ghalamfarsa et al. (209) confirmed that while *P. drechsleri* was monophyletic, the *P. cryptogea* complex formed 3 well-defined phylogenetic groups, with group I closely

affiliated with *P. erythroseptica* and groups II and III together on a basal clade. Some isolates were intermediate between groups II and III and exhibited a greater amount of heterozygous bases than the other isolates, suggesting possible outcrossing between these groups. Additional analysis including the holotype strain of *P. cryptogea* is needed to identify the clade representing the *sensu stricto* classification of this species as well as the correct taxonomic classification of the remaining clades and if hybridization between them is occurring. Using a parsimony-based ancestral recombination graph (ARG) and genealogies inferred from the β-tubulin and translation elongation factor 1-α genes, Olson et al. (219) suggested that divergence between *P. cryptogea* and *P. drechsleri* was recent and that speciation is still in progress, a possibility that deserves further examination.

P. citricola is another species complex where prior isozyme (221) and SSCP analysis (161) suggested multiple species, and more recent multigene phylogenetic analysis separated out the new species *P. plurivora* (151), *P. multivora* (247), *P. capensis* (24), *P. elongata* (228), and possibly other new species (151). While a recent taxonomic evaluation has re-elevated *P. pini* to a valid species description (120), there are still several taxonomic subgroups such as *P. citricola* clade E (120) and *P*. taxon *emzansi* (reviewed in Bezuidenhout et al., 24) yet to be fully characterized. Isozyme and molecular analysis confirm that other species such as *P. citrophthora* (201) and *P. capsici* (39,200) are complexes composed of more than one species. An effort to delineate species boundaries in a number of these and several other species complexes, as well as describe new species using multilocus phylogenetic analysis, is in progress in several labs and will hopefully provide better resolution that is needed in the not too distant future.

Characterizing hybrid populations. In the laboratory, interspecific hybrids have been generated between *P. capsici* and *P. nicotianae* by zoospore fusion (79) and transfer of isolated nuclei (106), and between *P. infestans* and *P. mirabilis* (99), *P. capsici* and *P. tropicalis* (70), and *P. sojae* and *P. vignae* (199) in sexual crosses. In the field, hybrids have been recovered for *P. nicotianae* and *P. cactorum* (37,128,189), *P. cactorum* and *P. hedraiandra* (187), and *P. cactorum* and *P. nicotianae* (*P.* × *pelgrandis*, 214). *P. andina* is a naturally occurring hybrid with *P. infestans* and an unknown species as the parents (101). Perhaps the most notable natural hybrid is *P. alni,* which is associated with alder dieback in Europe (42,65). The most common form is *Phytophthora alni* subsp. *alni,* while other variants are collectively known as *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*. While the parentage of these hybrids was thought to be species closely related to *P. cambivora* and *P. fragariae*, more recent analysis suggests that *P. alni* subsp. *alni* is derived from several hybridization events between *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis* (133). In addition to the characterized hybrids described above, "hybrid swarms" as described by Burgess et al. (47) may also occur in nature. Hybrid swarms among natural *Phytophthora* populations contain mixtures of parent, offspring, and intermediate types with high tendencies for backcrossing and outcrossing. Their work with clade 6 isolates recovered from native ecosystems in Western Australia identified different alleles of the ITS rDNA repeat unit in single isolates that also possessed a polymorphic *cox1* gene. They concluded that the presence of such hybrid swarms is indicative of sexual and somatic hybridization events; the high proportion of these variant isolates within the population also implied that these hybridization events were common.

Hybrid species represent a major challenge in the development of robust diagnostic protocols. This is especially true if the target for the diagnostic marker is encoded on the mitochondrial DNA, as this is uniparentally inherited from the maternal parent (86,291). Diagnostic protocols using species-specific PCR markers that include both a nuclear and mitochondrial locus may suggest the hybrid nature of an isolate if there is a lack of agreement between the two loci. Other techniques that allow for identification of multiple species at the same time, such as macro/micro arrays, SSCP, or sequence-based identification, are typically better at identifying hybrid isolates.

Isolation of *Phytophthora* spp.

Selective media for isolation usually consists of cornmeal agar (CMA) or clarified V8 juice agar (cV8) amended with a combination of antibiotics including pimaricin, ampicillin, rifampicin, vancomycin, nystatin, and fungicides pentachloronitrobenzene (PCNB) and hymexazol (also known as Tachigaren) (reviewed in Erwin and Ribeiro, 82). The selective activity of these media is mainly due to pimaricin or nystatin, both active against most Eumycotan fungi. The selective media are light sensitive and thus should be kept in darkness. Hymexazol inhibits most *Pythium* and *Morteriella* species (the only genus in Mucorales which contains species insensitive to pimaricin and other polyene antibiotics), which can overgrow and mask *Phytophthora* (276). There are several *Pythium* spp. that are resistant to hymexazol and some *Phytophthora* spp. that are sensitive; thus adjustment of the concentration might be required (7,108,118). For bacterial management, the combination of rifampicin and ampicillin was found to be more effective than vancomycin alone (145). The selective media "favors" the growth of *Phytophthora* but does not guarantee successful isolation. Important consideration should also be given to the quality (newer actively growing infection versus older infection) or type of sample material from which isolation is attempted (bark, leaf, soil, or water). For additional information on selective medium and techniques for isolation, see Mitchell and Kannwischer-Mitchell (208) and Erwin and Ribeiro (82).

Isolation from soil and water. A wide range of methods have been reported for the successful isolation of *Phytophthora* from soil and water samples, including soil plating or baiting methods. Due to the limited amount of soil that can be deposited on the agar surface, direct soil plating is typically utilized when *Phytophthora* inoculum is high and is not suitable for recovery of some species that give rise to few viable propagules per gram of soil, such as *P. cinnamomi* (117). Soil baiting has several advantages over direct plating. First, a larger volume of soil can be tested, increasing the likelihood of detecting species present at a low population density. Secondly, homothallic species that survive as dormant oospores are more likely to be detected by baiting than by direct plating (83). Soil baiting involves the flotation of zoospore-attractive baits in soil-water suspensions, typically requiring 2× the amount of distilled water as soil, or a lower water/soil ratio in cases where the inoculum level is low (18,150,274). Zoospores swim upward and colonize these baits, which are then washed under distilled or tap water (mainly to eliminate bacteria buildup on the bait surface), blotted dry, and sections with discoloration are placed in selective media. Success of isolation from soil is often increased when soil is kept between 15 and 20°C and the bait is not wounded, which discourages colonization by *Pythium* spp. and bacteria (83,92,130, reviewed in 82,144). False negatives can be avoided when soils are re-baited after air-drying, premoistening, or cold storage practices that induce germination of dormant spores (18,64,144,268). It should be noted that populations of *Phytophthora* spp. may fluctuate depending on the season (19,20,115,202,290), so samples may need to be collected at different times of the year to get an accurate picture of population density or for detection purposes. Leaf tissue for bait material has been preferred in most recent studies rather than apple, pear, or lemon fruit reported in historical records (82). A wide variety of plant foliage has been utilized (274), although not every type of bait is equally attractive to diverse *Phytophthora* spp. Leaves of *Camellia*, *Rhododendron*, and *Quercus* spp. have been more commonly used in recent years (18,84,92,150, 229,257,260). Leaves with little or no trichomes enable direct contact with water during the baiting routine. Observation of discoloration is best on young tissues such as leaflets, cotyledons, and radicles. These baits have another advantage over "leathery" baits by enabling the examination of sporangia under a light microscope.

Protocols involving slight variation in filtration and baiting techniques also exist for isolation of *Phytophthora* spp. from water samples with leaf or fruit baits being placed in a mesh bag and

flooded in water (e.g., stream) for a period of time. The retrieval time depends greatly on water temperature; with temperatures exceeding 15°C the infection process can be rather quick (3 to 7 days) (130). Foliage of evergreen plants such as rhododendron, *Ilex* sp., or *Lithocarpus densiflorus* with thick-leathery structure appears to be more suitable compared to brittle leaves that can be rapidly colonized by diverse organisms. However, no difference was found among evergreen leaves in baiting a diverse range of species in streams (229), although Ghimire et al. (92) reported that rhododendron leaves yielded the greatest diversity and population of species. In addition to baiting, 3 or 5 µm pore sized nitrocellulose or polycarbonate filters have been used to isolate *Phytophthora* spp. from water (121,130,229). If water is murky and contains large organic particles, prefiltering with cheesecloth or miracloth may be necessary. Once water is filtered, the filter is placed face-down on selective media and the plates are incubated for 1 to 3 days. Filters are then removed and the media examined daily for colonies typical for *Phytophthora* spp. and subcultured. Similar to direct soil plating, filtration of water samples can be done with a limited amount of water (50 to 200 ml per filter). Therefore, repeated and systematic sampling of streams and soil within an area will be needed to reveal the true biodiversity of *Phytophthora* spp. (131). The USDA-APHIS has an approved procedure for recovery of *P. ramorum* from water samples (http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/surveyplan/appendixI.pdf).

Isolation from plant tissue. Isolation of *Phytophthora* spp. is achieved from lesions without surface sterilization when infection is active and samples are taken from the advancing lesion margin (149,151,255,275). The success of isolation from necrotic tissues can be decreased by the presence of antagonistic bacteria and phenolic compounds produced by the plant that inhibit growth of *Phytophthora* spp. With plants that produce high amounts of phenolic compounds, such as *Alnus* sp., repeated washing and plating numerous infected tissue samples on selective media can increase the chances of isolation of the target *Phytophthora* spp. (124,149,148,255). Tissue samples should be blotted dry using filter paper before plating to discourage bacteria buildup. If surface disinfestation is attempted, particularly from tissue with advanced necrosis, 30 seconds of exposure to 70% ethanol (ethanol does not leave residue and there is no need for washing when compared to the use of sodium hypochlorite) followed by drying on filter paper appears to be sufficient. With evergreen plants, leaf colonization by saprophytic organisms is much slower compared to deciduous plants, thus providing a longer window for isolation. For example, *P. ramorum* was still recovered from older necrotic areas of inoculated evergreen foliage of rhododendron and bay laurel (*Umbellularia californica*) after 49 weeks (85).

Approaches for Morphological Identification

Identification of many *Phytophthora* species can be relatively straightforward; however, overlapping morphological features and intra-specific variability can make *Phytophthora* a difficult genus for species identification (82). It is important to start the process with clean cultures obtained using an appropriate selective medium (noted above). Placing a transfer plug with mycelium in a sterile petri plate and overlaying with a block of selective medium on top can be helpful to clean up most bacterial or fungal contaminants. The hyphae will grow through the medium toward the surface free of bacteria; subsequent transfer to nutrient agar can be used to verify if the isolate is free of bacterial contamination. Hyphal tips of clean cultures are then transferred and maintained commonly on media such as CMA, cV8, potato dextrose agar (PDA30; Difco using 30 g instead of the 37 g/liter indicated by the vendor), lima bean agar (LBA), carrot agar (CA), oatmeal agar, rye agar, and hempseed agar depending on the species (see Erwin and Riberio, 82). Another effective method to eliminate bacterial contamination is to inoculate a leaf (i.e., rhododendron) or fruit (i.e., apple, pepper) and isolate the organism from resulting lesions.

The growth medium of choice is particularly important for consistency in species description and identification purposes. Commonly utilized media include V8 juice agar for growth-temperature relationship and morphological features and CMA, cV8, and PDA30 for colony pattern. For evaluating sporangia morphology and size measurements, liquid culture should be utilized. For this purpose, a plug (ca. 0.5 cm^2) taken from the margin of an actively growing isolate is transferred to a new petri dish and flooded with tap water, distilled water, 10% sterile or nonsterile soil extract solution, pond water, rain water, or river water (82,88). Isolates can be incubated at room temperature on the lab bench, but under white fluorescent light they may exhibit more consistent production of sporangia (82,88). For some species, nonsterilized liquids or sprinkling nonsterile soil on water cultures can induce sporangial formation. Plugs are observed under a compound microscope after 12 to 24 h; if sporangia have not formed, remove the liquid and repeat the flooding and incubation (88). When measuring sporangia size, attention should be paid to whether they are fully developed (individual zoospores are recognizable within the sporangia cytoplasm) and whether they are the first produced sporangia. The size of secondary produced sporangia becomes smaller, particularly with species with nested proliferation (see below). To ensure that statistically supported measurements are obtained, the number of sporangia that are measured should be between 20 and 50. To determine the persistence (caducity) of sporangia, sporulating agar plugs of mycelium are removed from the liquid culture, placed in contact with a microscope slide containing a drop of water, and moved briskly to dislodge sporangia. If the species is caducous, many dislodged sporangia with pedicels will be observed under the microscope.

A listing of the 117 described species in the genus with their morphological features used in making taxonomic classifications are presented in Table 1 (29 of these are exotic and of concern to the USDA-APHIS regulatory personnel, 246). The most robust character to start the morphological identification of species is the thickness of papilla of the sporangium, classified as papillate (papilla size ≥3.5 µm) (Fig. 2A to D), semipapillate (papilla size <3.5 µm) (Fig. 2E to G), and nonpapillate (Fig. 2H) with very slight apical thickening (Table 1). A "natural arrangement" of the species by the type of papilla, with some exceptions, is observed in the groupings of a phylogenetic analysis (32,165,195,196). Clades 1 to 5 include papillate and semipapillate species; Clades 6, 7, 9, and subclade 8a contain nonpapillate species; Subclades 8b, 8c contain a mix of semipapillate and nonpapillate; and Clade 10 contains a mix of papillate and the nonpapillate species. Another useful feature for identification is the caducity of the sporangia and the length of the pedicel (short: less than 5 µm [Fig. 2B and E], medium: between 5 and 20 µm [Fig. 2C and F], or long: greater than 20 µm [Fig. 2D and G]). Almost all nonpapillate species have persistent sporangia (Fig. 2H) except *P. pinifolia,* which is partially caducous with a medium pedicel (75), and *P. foliorum,* which is partially caducous with a short pedicel (69). Another consistent character for identification is the proliferation of the sporangia (internal, external, or nested [Fig. 2I and J]). Most papillate and semipapillate species do not show internal proliferation except *P. multivesiculata* (132) and *P. plurivora* (151). Most nonpapillate species have proliferating sporangia except *P. rosacearum* (109), *P. pinifolia* (75), *P. primulae* (263), and *P. quininea* (62). Sporangial shape (ovoid, obovoid, pyriform, obpyriform, clavate, obclavate, reniform, irregular), size (length-breadth ratios), and base (tapered or rounded) are other important features for identification (82,88). Most *Phytophthora* spp. show variation in sporangia shape; thus the most common types should be identified and measured. Branching of sporangiophores (unbranched, with globose swellings, close sympodial, simple sympodial, compound sympodial, umbellate, and irregularly branched) in liquid cultures or agar needs also to be noted for identification (Fig. 2K to N). Sporangiophore shape is not a very informative character for identification, with the exception of the umbellate type sporangiophores that are only produced by the papillate species *P. tropicalis* (11) (Fig. 2N) in Clade 2a and *P. morindae* (212) in Clade 10. The semipapillate

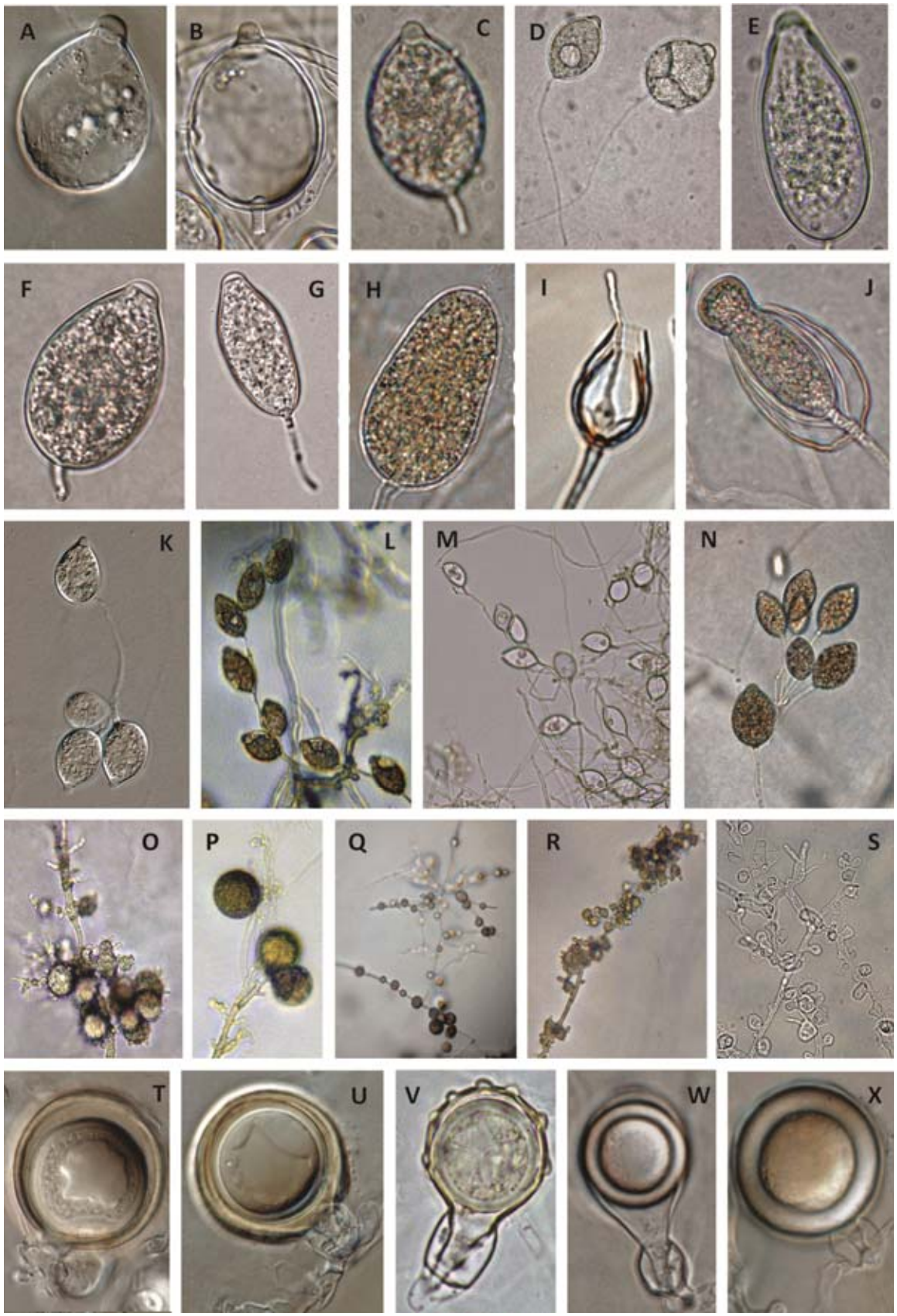

Fig. 2. Morphological features of *Phytophthora* species asexual and sexual stage. **A to H,** Sporangia papilla and pedicel. **A to D** = Papillate: **A,** *P. idaei* (persistent); **B,** *P. boehmeriae* (short); **C,** *P. kernoviae* (medium); **D,** *P. capcisi* (long). **E to G** = Semi-papillate: **E,** *P. ramorum* (short); **F,** *P. nemorosa* (medium); **G,** *P. hibernalis* (long). **H** = Non-papillate *P. cinnamomi* var. *parvispora*. **I and J,** Sporangium proliferation. **K to N,** Sporangiophore. **K to M** = Simple sympodial: **K,** *P. boehmeriae*; **L,** *P. ramorum*; **M,** *P. kernoviae*; **N,** Umbella Type *P. tropicalis*. Chlamydospores: **O,** *P. cinnamomi*; **P,** *P. ramorum*. Hyphal swellings: **Q,** *P. drechsleri*; **R,** *P.* taxon *kelmania*; **S,** *P. foliorum*. Paragynous antherium: **T,** *P. tentaculata*. Amphyginous antheridium: **U,** *P. idaei*. Wall projections: **V,** *P. cambivora*. Oogonium with tapered base: **W,** *P. hevea*. Oospore with thick wall: **X,** *P. kernoviae*. Photos by Z. G. Abad, USDA-APHIS-CPHST.

P. hibernalis produces irregular sympodium and occasionally umbellate sporangiophores.

Chlamydospores are thick-walled, long-term survival spores delimited from the mycelium by septa. Presence (or occasional presence), position (lateral, terminal, or intercalary), and shape (globose, subglobose, or elongate) are diagnostic features of chlamydospores that support identification (Fig. 2O and P). In contrast, hyphal swellings are thin-walled bodies not delimited by septa and may be variable in shape. The presence (or occasional presence), shape (globose, subglobose, elongate, obovate, obpyriform, distorted shapes, catenulate, radiate, and clustered), position (terminal and intercalary), and formation of chain-like clusters are features useful in identification (Fig. 2Q to S). Although hyphal swellings are not strong characters for species differentiation, they can be useful in the identification of some species such as in the *P. cryptogea*/*P. drechsleri* "complex".

Determination of the sexual stage of *Phytophthora* is the second most important morphological feature and involves the production of the oospores through the fusion of the oogonium and antheridium (maternal and paternal gametangia, respectively). Homothallic species produce oogonia in single cultures, while heterothallic species produce them in the presence of a strain of the opposing compatibility type (A1 or A2). The addition of sterols to the growth medium was originally considered to be essential to sexual reproduction in pythiaceous fungi (77,116); however, several fatty acids, including palmitoleic, followed by oleic, palmitic, and linoleic, also stimulate oospore formation in certain species of *Phytophthora* (143,159). We have successfully used UDO Oil (made with organic flax, sesame, and sunflower seed oils; Flora, Inc., Lynden, WA, USA) at a rate of 200 µl/400 ml in hempseed agar-based media to stimulate oospore production at 20°C in darkness (Z Gloria Abad, *unpublished*). For the production of the sexual stage on agar medium, a mycelial plug of the unknown isolate is cultured individually in LBA to determine if it is homothallic. If oospores are not produced, the unknown culture is placed on one side of the agar plate and a plug of a tester strain (A1 or A2) on the other side. Intra- and interspecific pairings with testers (A1 and A2), including *P. cambivora*, *P. capsici, P. cryptogea, P. drechsleri, P. meadii*, *P. nicotianae, P. tropicalis,* and other heterothallic species, can be used. A number of media noted above amended with the above-mentioned fatty acids are appropriate for mating type tests. The polycarbonate membrane method described by Ko (157) facilitates identification and genetic studies of heterothallic species of *Phytophthora*. The sexual structures can be formed in interspecific pairings across the polycarbonate membrane by hormonal influence of the opposing mating type (157,158). The sexual stage usually forms in the dark and at temperatures lower than the optimum temperature for growth (e.g., 20°C). Production of oospores can occur in approximately one week up to months depending on the species. The use of agarose instead of agar is reported to favor the production of increased numbers of oospores (157).

Of the 117 reported *Phytophthora* species, the majority are homothallic (Table 1). A few nonpapillate species are self-sterile, and the production of their sexual phase has not been observed; these include *P. litoralis* and *P. pinifolia* in Clade 6 (75,154), *P. machrochlamydospora* and *P. quininea* in Clade 9 (62,138), *P. gallica* in Clade 10 (153), and *P. gemini* (188). There are no papillate or semipapillate species reported to be self-sterile. *P. gonapodyides*, *P. thermophila*, and *P. lateralis* are self-sterile or can produce gametangia under special conditions (10,45,154,277). *P. leersiae* is the only species reported to produce only the sexual stage (119). Some heterothallic species (i.e., *P. capsici*) can occasionally be self-fertile in single culture (especially in fresh isolates without many transfers; Z. Gloria Abad, *personal communication*).

Antheridia in *Phytophthora* can be either paragynous or amphigynous. A paragynous antheridium is formed when the antheridial hyphae oppresses laterally to the oogonial stalk (Fig. 2T). An amphigynous antheridium is formed when the oogonial hyphae grows through the antheridial hyphae forming a kind of collar that surrounds to the antheridial stalk (Fig. 2U). Some species produce consistently one type of antheridia, whereas several species produce both types and can be predominantly amphyginous or predominantly paragynous (Table 1). Almost all heterothallic species have amphigynous antheridia except *P. hydropathica,* which produces both types of antheridia (122). *P. japonica,* described by Waterhouse (288), was reported to be a heterothallic species that produced both amphigynous and paragynous antheridia, but the type culture of this species was lost. A few species may have multiple antheridia, such as *P. glovera,* which produces both paragynous and amphigynous types on a single oogonium (2). *P. insolita* is the only species of *Phytophthora* where no antheridium is seen despite the production of oospores.

A few *Phytophthora* species have ornamented oogonial walls (Fig. 2V), including *P. alni* subsp. *alni*, subsp. *multiformis*, and subsp. *uniformis* (44), *P. cambivora*, *P. katsurae*, and *P. cyperibulbosi* (82). Some species produce oogonia with tapered bases (Fig. 2W; Table 1). Oospores can be plerotic if the oogonium is filled (Fig. 2X), or aplerotic if there is space left between the oospore wall and the oogonium (Fig. 2T). Some species, like *P. kernoviae* and *P. morindae,* have very thick walls at early stages (Fig. 2X) that become thinner with the maturation of the oospore. The oospore shape is not a strong character for species identification. The optimum and maximum temperature for growth can be additional information to support the identification of species (Table 1).

Although *Phytophthora* is one of the most studied genera of plant pathogens, there is limited information in international herbaria and culture collections on the accession numbers used for the types (Holotype = dry specimen; Ex-holotype = original culture). Likewise, publications on morphological or molecular taxonomy do not always include this information. Although considerable advances in molecular systematics have been made in the last 10 years, there is still confusion when identifying isolates of some described species, recognizing new species, and clarification of species complexes. This confusion is due in part to the number of sequences in GenBank from misidentified cultures or poorly annotated sequences as well as a lack of an ex-holotype culture in the analysis. The "Type" or "Holotype" defines the species, and if this primary specimen is lost, the information for the Lectotype or Neotype (representative of species when original description based on syntypes or when the holotype is no longer available, respectively) is fundamental for the correct recognition of species. In order to develop accurate systematics (taxonomy, nomenclature, and phylogenetics), it is very important that the rules established by the International Code of Nomenclature for algae, fungi, and plants (ICN) Melbourne Code 2011 (formerly International Code of Botanical Nomenclature [ICBN]) are followed and that the importance of the Types are recognized (1).

Approaches for Molecular Identification

A variety of molecular techniques have been used for species- and subspecies-level identification ranging from the technologically complex to simpler procedures requiring less technical skill or sophisticated equipment.

Sequence-based species identification. The most accurate molecular method for identification of isolates to a species level is accomplished by sequence analysis of specific loci. Historically, the ITS region has been used, and since there is a large dataset for this locus that includes all described species, it continues to be a mainstay for this purpose. However, this is not the optimal locus for all species, particularly those that are phylogenetically closely related (for example, *P. rubi* and *P. fragariae* have identical ITS sequences; some Clade 1c species cannot be differentiated with this locus). More recently, a number of alternative nuclear (60S ribosomal protein L10, β-tubulin, enolase, HS protein 90, large subunit rRNA, TigA gene fusion, translation elongation factor 1α; 32,165,283) and mitochondrial (*cox1, nad1, cox2, nad9, rps10,* and *secY*; 165,191,193,195,196) loci have been sequenced for phylogenetic resolution within *Phytophthora* that are also useful for identification purposes. Background information for amplification

and sequencing of many of these loci, as well as the capability for BLAST searches against a curated database for isolate identification, may be found at the *Phytophthora* Database (www.phytophthoradb.org). When conducting BLAST analyses, it is important to use a curated dataset to ensure sequences reflect accurate species classification. A dataset for ITS and *cox1* and *2* spacer sequences is also available at *Phytophthora* ID (105) (www.phytophthora-id.org) and sequence data for several loci (ITS, β-tubulin, elongation factor 1α, or *cox1*), along with pictures of morphological features, may be found at Q-Bank (www.q-bank.eu). The ITS region, along with a portion of the *cox1* gene, have also been proposed as the two loci to include in the Barcode of Life Database (www.boldsystems.org), and representative sequences for all described and some provisional species have been deposited (232). To support the classification of a new species, it is important that sequences from several of the above-noted loci are generated.

While BLAST analysis can be an effective tool for identifying isolates to a species level, there are several considerations to keep in mind to ensure sequence-based results are accurate (155). BLAST scores are commonly used to evaluate classification of isolates, but since these scores are dependent on the length of the aligned sequences as well as the amount of sequence identity, there will be instances where high levels of sequence identity for only a portion of the target sequence may provide an incorrect impression of species classification. Also, it is common to encounter results where the scores are similar for comparisons with different species, which makes it difficult to draw conclusions on species identification. While it is possible that the unidentified isolate may represent a new species, if the locus is known to exhibit intraspecific polymorphisms it could also represent an example where clear delineations among closely related species cannot be made with this region. For identification of *Trichoderma* spp. using the ITS region, this problem was addressed by determining which regions were invariant at the intraspecific level and using only those for BLAST analysis (72); with the extent of the ITS dataset available, this approach could also be employed with *Phytophthora*. Heterozygosity may also complicate efforts at identification with nuclear loci, and while this may be reflective of outcrossing of a diploid organism (e.g., *P. capsici*), it may also represent an interspecific hybrid (having the two alleles group separately in phylogenic analysis would be indicative of this). It also is important to recognize that the taxonomic classification of the genus has not yet caught up with phylogeny; there are species complexes with closely related species where morphological features alone are not enough to delineate species boundaries observed from phylogenic analysis (examples discussed above). Until these complexes are subjected to detailed multilocus analyses using isolates representing the diversity observed in nature, identification by sequence analysis alone will continue to be problematic for some species. Comparing results from several loci is necessary to confirm species identification, especially when working with species complexes.

Gel-based molecular techniques for species identification. There are several gel-based techniques that can be used to identify isolates to a species level using amplicons generated by PCR. Ristaino et al. (231) observed that amplification of the ITS region followed by digestion with restriction enzymes generated restriction profiles that could be useful for identification to a species level. The use of PCR-restriction fragment length polymorphism (RFLP) of the ITS region for species identification was expanded by Cooke et al. (59). Using this approach, Camele et al. (51) characterized isolates from Italy, Bowman et al. (40) differentiated *P. palmivora* from *P. nicotianae*, and, coupled with sequence analysis of the ITS region, Roy et al. (233) examined *Phytophthora* spp. in Eastern India. Drenth et al. (71) improved the technique by developing primers that were specific for *Phytophthora*, allowing amplifications to be done directly from environmental samples.

In addition to the ITS region, PCR-RFLP analysis of the *cox1* and *cox2* gene clusters has been found useful for species identification (197). While this technique cannot be used with DNA extracted from infected plant tissue (the amplification primers are not specific for *Phytophthora*), another primer pair (Phy-8b and Phy10b) spanning the spacer region and containing portions of the flanking *cox1* and *cox2* genes is specific for amplification of *Phytophthora* spp. (they do not amplify plants or the closely related genus *Pythium*, 198) and hence can be used for amplification from infected plant tissue. When the pathogen DNA concentration is low it may take two rounds of amplification to observe a visible band in a gel. The amplicon size varies among species but is generally less the 0.5 kb, and single digestion with *Apo*I and *Dra*I can differentiate many but not all species (F. Martin, *unpublished*).

SSCP analysis of the ITS region of the rDNA is another gel-based method found useful for species identification (161). The ITS-1 region is amplified, denatured, and separated on a nondenaturing polyacrylamide gel with the resulting banding profiles useful for species identification. A technique for direct colony amplification of the ITS-1 region has been published (163). The spacer region between the *cox1* and *cox2* genes has also been used with this technique (229). An overview of SSCP migration patterns for 60 species in the genus *Phytophthora*, along with morphological keys and photomicrographs, may be found in Gallegly and Hong (88). The technique has been modified by Tom Kubisiak using an automated sequencer for data collection, thereby improving accuracy of the data and simplifying its analysis and comparison among isolates (reviewed in Martin et al., 194; the online eXtra of this cited article has the techniques). Ghimire et al. (92) used SSCP to identify species recovered from irrigation runoff containment basins.

Molecular techniques for population analysis. Numerous techniques have been employed for studying the population biology and diversity of *Phytophthora* species, including RFLPs, random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), microsatellites, single nucleotide polymorphisms (SNPs), and mitochondrial haplotype analyses.

RFLP analysis using Southern blots of digested genomic DNA probed with specific cloned nuclear DNA fragments has been useful for identification of subpopulations of several species (86,98,183). RFLP analysis of purified mitochondrial DNA (mtDNA) in agarose gels also has been useful for identification of species as well as subpopulations (reviewed in Erwin and Ribeiro, 82), but is time-consuming due to the effort required for mtDNA purification. With the high copy number of mtDNA, digestion of total DNA with restriction enzymes that cut in GC-rich regions can sometimes be useful for generating discrete bands for differentiating isolates (170,291). While these approaches for identification of species and subpopulations can be useful, the techniques described below are more suitable for this purpose.

RAPDs generate dominant markers that can provide a large number of polymorphic bands without having knowledge of genomic DNA sequences. It has been useful for analysis of *P. capsici* (139), *P. cinnamomi* (182), *P. infestans* (4), *P. nicotianae* (298), the relationship among Clade 1 species (60), *P. colocasiae* (206), and looking at genotypic diversity of several species in eastern India (233) and China (300). One main limitation of this marker system is the reproducibility of results between different labs and even between different thermalcyclers. One approach to alleviate this problem is to only use primers that generate robust bands, although a better approach is to generate sequence characterized amplified region (SCAR) markers by cloning the bands and designing primer pairs for their amplification (this also eliminates the need for using the same concentration of DNA for all samples as required for RAPD analysis).

AFLP analysis is another technique that generates dominant markers for population analysis where there is little knowledge of genomic DNA sequences. The technique can generate a large number of markers, is relatively straightforward, and is more transferable among labs than RAPDs. AFLP analysis has been used for comparison of isolates of *P. andina* (218), *P. cactorum* (26), *P. capsici* (39,172), *P. cinnamomi* (73), *P. drechsleri* (171), *P. infestans* (4), *P. nicotianae* (171,297), *P. pinifolia* (74), and *P. ramorum* (141).

Simple sequence repeats (SSRs), generally referred to as microsatellites, are also commonly used in population analysis and assessment of intraspecific variation. This method is better to use than RAPDs or AFLPs due to the loci being codominant as well as having a higher degree of reproducibility among labs. The major drawback, however, is the need for genomic DNA sequence data to identify the location of potentially informative SSRs and design primers for their amplification. With a wider range of species undergoing genomic sequencing, data from more species should be available in the future. Another approach for generating the necessary sequence data without analyzing the entire genome is sequencing genome survey sequence libraries (5). SSR analysis has been successfully used with *P. cinnamomi* (67,68), *P. infestans* (175,292), *P alni* (134), and *P. ramorum* (100,102,140,225, 226,280,281). When conducting population analyses, it is advisable to clone and sequence representative amplicons of each size class to ensure size differences are due to varying numbers of simple sequence repeats and not from length mutations in flanking regions. Stewart et al. (254) observed that capillary electrophoresis techniques were more accurate than gel electrophoresis for classifying SSR bands. Efforts to develop amplification primers for SSR loci for multiple species have been met with limited success (90,175,237). A modification of this technique is called intersimple sequence repeat analysis and uses a single primer based on multiple copies of a simple sequence repeat (e.g., $(CAT)_6$); when this sequence is close enough on opposite strands of DNA, a band is amplified. This has been useful for population analysis of *P. cactorum* (110–112), *P. citrophthora* on citrus in Corsica (57) and Spain (8), as well as *P. infestans* (156).

Single nucleotide polymorphisms (SNPs) can also be used to study pathogen subpopulations as well as for development of diagnostic molecular markers for their detection. For example, Bilodeau et al. (30) identified two SNPs each in the β-tubulin and CBEL (cellulose binding elicitor lectin) genes that differentiated the North American from the European populations of *P. ramorum* (a primer extension assay for rapid identification of the genotypes was subsequently developed). A more comprehensive analysis of this approach was recently completed by Abbott et al. (3) for *P. infestans* using SNPs in the flanking regions of microsatellite loci. Nucleotide diversity averaged 1 SNP per 426 bp, and while heterozygosity precluded the use of many SNPs, a number of homozygous loci were identified that could be used for genotyping isolates. From examining a subset of unlinked loci, the authors concluded that 7 loci had a high probability of correct identification of genotypes (99.9%). SNP analysis has also been useful in population studies with *P. capsici* (94,95,127). In these latter studies, melt curve analysis rather than DNA sequencing was used to identify the SNPs. Mitochondrial loci can also be useful for identification of SNPs that differentiate subpopulations, but it must be kept in mind that in a sexually outcrossing population, the mitochondrial background would not reflect nuclear genotypic differences. SNPs in the *cox1* gene and *cox1* and *cox2* spacer region capable of differentiating the North American and European lineages of the clonally reproducing *P. ramorum* have been identified (78,167,191).

Mitochondrial haplotypes identified using RFLP analysis have been useful for population studies of *P. infestans* (reviewed in 91,289), and the sequence data thus far suggest this method may be useful for differentiating haplotypes for a wide range of *Phytophthora* spp. Schena et al. (239) examined several mitochondrial regions they felt would be useful for finding interspecific polymorphisms in a wide range of species and with the *trnY-rns* spacer region observed intraspecific variation with a limited number of species where multiple isolates were examined. Using a comparative genomics approach with mitochondrial genome sequence data from NA-1 and EU-1 genotype *P. ramorum* isolates, sequences from 7 loci from a range of isolates identified 4 mitochondrial haplotypes among the 3 nuclear genotypes (191). More recent analysis of *P. cinnamomi* using 7 loci identified 45 mitochondrial haplotypes across 62 isolates (193), while there were 20 haplotypes for 51 isolates of *P. nicotianae* primarily from Italy (186). A multilocus analysis of the genus using four mitochondrial genes is currently in progress (Fig. 1); of the 64 species where multiple isolates had all four loci sequenced, 69% exhibited multiple mitochondrial haplotypes. Since these regions are predominantly coding regions rather than spacer regions, where many of the polymorphisms were identified in *P. cinnamomi,* it is likely that addition polymorphisms will be identified with further analysis. To facilitate this effort, the mitochondrial genomes of a number of *Phytophthora* species have been sequenced and comparative genomics employed to identify specific regions associated with genome rearrangements, as well as spacer regions that may be useful for haplotype classification (F. Martin, *unpublished*).

Diagnostic Techniques

Immunodetection. The enzyme-linked immunosorbent assay (ELISA) is a biochemical diagnostic tool based on antibodies that recognize an antigen specific to a plant pathogen. Commercially available ELISA tests for *Phytophthora* detection exist in a number of different formats, including assays for high throughput use in multi-well plates, and ImmunoStrips or Lateral Flow Devices (LFD) for single-use, on-site diagnostics. Immunodetection of *Phytophthora* in various types of plant related samples has been recently adopted by a broad range of plant professionals, mainly due to test availability, simplicity, efficiency, cost, and speed with which results are produced. Because the ImmunoStrip and LFD tests do not require special lab equipment, these formats are particularly suited for users with limited technical resources and experience.

ELISA-based assays typically utilize antibodies that detect a generic *Phytophthora* antigen and are not species specific (6,184). The antibodies of some commercial ELISA tests are also known to react with some species of *Pythium*, yielding false-positive results. Numerous comparative studies involving the detection of *Phytophthora* using ELISA, plating, and/or PCR-based methods have highlighted differences between each test's performance, although the majority of samples testing positive by PCR and/or isolation have also tested positive by ELISA (46,164,217,220). Since antigens can be present in tissues even after the pathogen is no longer viable, ELISA can be used for diagnostics even after certain treatments have been applied to control the disease, such as the application of fungicides or thermal inactivation.

ELISA-based protocols have successfully detected *Phytophthora* spp. in numerous types of samples, including plant foliage (173), plant roots (22,184), soil (244,262,294), irrigation water (6,49,50), and hydroponic nutrient solutions (103). In certain cases, these assays have not detected the presence of the pathogen, yielding false-negative results. Failure to detect the antigens may be related to the type or quality of the host tissue and/or the particular *Phytophthora* species. For instance, some types of plant material may be difficult to macerate in order to release the antigens; one study reported a decrease in false-negative results by adding cutting, tearing, or grinding steps during sample preparation (173). In addition, the sensitivity of detection of different ELISA tests can vary in regard to the *Phytophthora s*pecies, and even within isolates of the same species (6).

Species-specific antigens within the genus *Phytophthora* have yet to be identified, although more specific serological tests with higher sensitivity to particular *Phytophthora* species have been investigated (14). Antibodies with highest sensitivity and specificity to *P. ramorum* and *P. kernoviae* have been produced, although these antibodies were also found to be cross-reactive with other *Phytophthora* species. While species identification is not directly possible using ELISA-based assays, DNA on *Phytophthora*-positive ImmunoStrip and LFD nitrocellulose wicks can be amplified by adding a section of the membrane directly to an amplification reaction, such as loop mediated isothermal amplification (LAMP) or ITS amplification for sequence-based species identification. *P. palmivora* (272), *P. ramorum,* and *P. kernoviae* (265) have all been specifically detected in plant samples using this novel approach.

ELISA-based assays are broadly adaptable. In certain cases, they have been utilized as a "prescreen" to reduce the number of samples needing further testing by more expensive and time-consuming diagnostic procedures for the confirmation of *Phytophthora* pathogens in large sample volume studies. In 2006, the USDA-APHIS approved a high throughput ELISA protocol as a primary screening tool for national *P. ramorum* surveys (46). The confirmation of *P. ramorum* within ELISA-positive samples is further required by PCR and/or culture plate isolation. ELISA procedures have also been utilized in multiple research studies to quantify the extent of *Phytophthora* infection (103,244,262), as the intensity of color in a positive reaction is proportional to the amount of pathogen antigen within the original sample. Grote and Gabler (103) reported that ELISA was easier to perform and more selective than the most-probable number method for quantification of *P. nicotianae* when comparing treatments for controlling this pathogen in hydroponic tomato systems.

Molecular techniques for identification and detection. The development of the PCR technique for amplification of DNA has revolutionized molecular diagnostics and enabled highly specific pathogen detection from small amounts of plant tissue. With properly designed amplification primers, highly specific diagnostic assays can be developed using conventional PCR techniques. One drawback, however, is the need for running samples on an agarose gel to visualize the amplified band diagnostic of the pathogen in question, which can increase the time required for sample analysis, does not allow for quantification of target DNA, and is an impediment to high throughput sample processing. Greater sensitivity and rapid sample processing can be obtained with real-time PCR, where a dye included in the amplification mixture fluoresces in proportion to the amount of DNA template that has been amplified. This fluorescence is quantified by the thermal cycler during the amplification, thereby reducing the time needed to obtain results, but does increase the cost of equipment and supplies to run the assays. The ability to use 96 well plates also enables high throughput analysis of samples. There are several different chemistries used for real-time PCR. SYBR Green is a dye that fluoresces only when it has intercalated in double-stranded DNA, and melt curve analysis is then used to confirm if the appropriate target sequence has been amplified. Having highly specific primers and an amplification devoid of other nonspecific amplification products (including primer dimers) is essential for obtaining accurate results. In general, a greater level of specificity can be obtained using TaqMan probes, where a target specific oligonucleotide has a fluorochrome at one end and a quencher molecule at the other. When the probe anneals to the template and is incorporated into the double-stranded DNA, these molecules are separated and the dye fluoresces. There are also other technologies, such as Molecular Beacons and Scorpion primers, that work on a similar principle as the TaqMan assay where there is no fluorescence signal when the fluorochrome is in proximity to a quencher molecule. One advantage of Scorpion primers is that they can be run with shorter cycling times, thereby reducing the time needed for obtaining results (264). In general, these assays are more specific than SYBR Green due to the need for sequence identity in the labeled probes. In contrast to these technologies, with the Plexor Q-PCR system, one of the probes is labeled with a fluorochrome, and as the DNA is replicated a quencher molecule is brought into proximity, suppressing fluorescence, resulting in a decrease in fluorescence during template amplification. Another advantage of using technology that utilizes a fluorochrome and quencher combination compared to SYBR Green is the ability to multiplex diagnostic markers by using a different fluorochrome for each target, thereby allowing as many as five templates to be detected in a single amplification. However, with some combinations of primers and probes, there can be a reduction in the amplification efficiency of templates when multiplexed, so this needs to be evaluated prior to deploying the assay.

Loop mediated isothermal amplification (LAMP) (215) is another technique for amplification of pathogen template for diagnostic purposes. Four primers are designed to anneal to different regions of the target, and DNA polymerase with strand displacement capability is used to amplify the template when incubated at a constant temperature (reviewed at Eiken Chemical Company Genome site: http://loopamp.eiken.co.jp/e/index.html). Amplified product can be detected visually due to turbidity of the amplification mixture (magnesium pyrophosphate is generated as a byproduct of the amplification). Dyes that intercalate into the double-stranded DNA (ethidium bromide, PicoGreen, EvaGreen) can be added to the amplification and fluorescence quantified upon excitation with UV light or when run in a real-time PCR thermal cycler (264). One advantage of this technique is that expensive equipment is not required since the temperature is not cycled during the amplification process. A detection system based on this technology has been developed for *P. ramorum*, but the sensitivity of detection was less when compared to a standard ITS TaqMan assay (10 pg compared with 250 fg, respectively) and there was background detection of *P. lateralis* (264). A rapid diagnostic technique has been developed that utilizes a lateral flow device as a primary serological screen for the possible presence of a *Phytophthora* spp., with a piece of the nitrocellulose membrane containing pathogen DNA placed directly in a LAMP amplification mixture, thereby eliminating the need for DNA extraction (266).

Array technology. An alternative approach for identification of multiple pathogens or species that are present in a sample is the use of array technology (macro- or microarray). Highly conserved primers are used to amplify a diagnostic region from a wide range of organisms; this is labeled and then hybridized onto an array of species-specific oiligonucleotides. The particular positions where hybridization takes place identify which species are present in the sample. One big advantage of this technique is that the ability to include positive controls to ensure amplification of target sequences in the extracted DNA has occurred (for example, including genus or clade specific oligonucleotides) as well as including oligonucleotides from multiple loci and in duplicate positions on the array to confirm results. While this technique can be very effective for identifying which pathogens are present in a sample, it may not be amenable for high throughput analysis of multiple samples due to the need to use one array per sample and the cost associated with this (although the nitrocellulose membranes of macroarrays can be stripped and reused). In view of the broad range of species that can be included on the array, this technology does provide an interesting platform for community analysis as has been done with *Pythium* (176,259). For a review on the use of array technology for detection of pathogens, see Lievens and Thomma (179).

Lievens et al. (178) evaluated the position of base mismatches in oligonucleotides derived from ITS sequences of *P. nicotianae* used for spotting on the nitrocellulose membrane, whereas Anderson et al. (9) looked at melting kinetics of positive hybridizations to optimize species specificity for detection of a range of *Phytophthora* spp. and confirm if the pathogen was correctly identified. Zhang et al. (296) developed a macroarray for identification of fungal and oomycete pathogens of potato based on the ITS region of the rDNA that included a positive control for fungi and potato tissue as well as from two to five pathogen specific oligonucleotides for identification of several genera of pathogens (including *P. capsici, P. erythroseptica, P. infestans,* and *P. nicotianae*). A *Phytophthora* species-specific macroarray has been developed that utilizes species-specific oligonucleotides from the *cox1* gene, the spacer region between the *cox1* and *cox2* genes, and the ITS region for genus and species-specific detection (W. Chen, Z. R. Djama, M. D. Coffey, F. N. Martin, G. Bilodeau, L. Radmer, G. Denton, and C. A. Lévesque, *personal communication*). While some cross reactivity was observed with the *cox1* oligonucleotides, the combination of all three loci allowed for correct identification of 82 out of 98 species tested (and 8 of 15 yet to be described species). Rather than using oligonuclotides as hybridization probes on the array membrane, Izzo and Mazzola (142) used the entire ITS region. While this reduced specificity (cross reactivity was observed for *Pythium* spp. that had less than 5 to 10% sequence divergence), it simplified

array construction and allowed for the detection of a wider range of soil fungi. With the increased amount of sequence data available for *Phytophthora* and related genera, it is likely that arrays will be more available in the future.

Another approach to array technology for generating templates for real-time PCR is a technique called padlock probes (258). In this technique, an oligonucleotide is developed that consists of two universal primers, a unique sequence for the probe called a ZipCode sequence, and terminal sequences that are capable of annealing to adjacent complimentary sequences of the target organism. When the proper target sequence is present and the termini anneal, they are ligated together to circularize the probe, which allows the two universal primers to then amplify the circularized oligonucleotide. The unique ZipCode sequence is used to develop a probe for real-time PCR detection and quantification (279) or a hybridization oligonucleotide for an array (258). Using this approach, it is easier to develop multiplexed assays for a range of plant pathogens; *Phytophthora* genus-specific and several species-specific markers have been developed (258,279). The use of 23 padlock probes specific for 22 *Phytophthora* spp. in a single microarray format was recently reported (36).

Massively parallel sequencing. On the forefront of diagnostics for a range of pathogens, including *Phytophthora*, is an approach known as massively parallel sequencing (MPS), which utilizes pyrosequencing (sometimes referred to as 454 sequencing). MPS is most commonly used to generate genomic sequences, but the technology can be augmented to detect and genotype plant pathogens in an environmental sample (190,256). Because MPS generates very large numbers (millions) of overlapping short sequence reads for all DNA in a sample, these data contain both host and pathogen sequences. A sequence sample database (SSD) from representative pathogen isolates will need to be developed by identifying a minimum number of highly diagnostic sequences, also called "electronic probes" or "E-probes", that are conserved among each of the target strains or species. These sequences must not be found in the genomes of plants or other common plant endosymbionts (236). This technology is currently being examined for use in diagnostics for regulatory purposes (http://bioinfosu.okstate.edu/MPS_site/people.html).

Other technologies. Tooley et al. (270) reported a ligase chain reaction for improving the specificity of detection of *P. infestans* using the ITS region of the rDNA. A thermal stable ligase was used to join two primers that annealed adjacent to each other on the target DNA, and the larger fragment is then visualized on a gel. By labeling the different primers with biotin or digoxigenin and using paramagnetic beads for separation, detection and quantification can be done by ELISA. A similar approach for detection and quantification of digoxigenin-labeled amplicons by ELISA was also used by Bailey et al. (15) for species-specific detection of several *Phytophthora* and *Pythium* spp.

Genus-specific diagnostic markers. While the ability to have a species-specific diagnostic marker is important, especially for a

Table 2. Selected references for species-specific diagnostic markers for detection of various *Phytophthora* spp.[a]

Species	Assay type	Locus	Reference
Phytophthora alni	Conventional	SCAR	Bakonyi et al. (16), De Merlier et al. (66), Ioos et al. (135)
P. boehmeriae	Conventional	ITS	Shen et al. (248)
P. cactorum	Conventional	SCAR	Causin et al. (52), Lilja et al. (181)
	Conventional	ITS	Bhat and Browne (25), Boersma et al. (33), Lacourt et al. (168)
	Real time	*Ypt1*	Schena et al. (239)
P. cambivora	Conventional	SCAR	Schubert et al. (245)
		ITS	Boersma et al. (33)
	Real time	*Ypt1*	Schena et al. (239)
P. capsici	Conventional	ITS	Silvar et al. (250), Ristiano et al. (231), Zhang et al. (299)
	Real time	ITS	Silvar et al. (249), Pavón et al. (224)
P. cinnamomi	Capture probe	*Cina*	Coelho et al. (56)
	Conventional	ITS	Boersma et al. (33), Williams et al. (293)
		Lpv	Kong et al. (162)
		SCAR	O'Brien (216)
	Real time	*Ypt1*	Schena et al. (239)
P. citricola	Conventional	ITS	Schuburt et al. (245)
	Real time	ITS	Böhm et al. (34)
		Ypt1	Schena et al. (239,240)
P. citrophthora	Conventional	SCAR	Ersek et al. (81), Goodwin et al. (97)
	Real time	ITS	Ippolito et al. (137), Schena et al. (241)
P. colocasiae	Conventional	ITS	Mishra et al. (207)
P. cryptogea	Conventional	*Ypt1*	Minerdi et al. (205)
		ITS	Boersma et al. (33)
	Real time	*Ypt1*	Schena et al. (239)
P. erythroseptica	Conventional	ITS	Nanayakkara et al. (210), Tooley et al. (267)
	Real time	ITS	Cullen et al. (63)
		rpb1	Atallah and Stevenson (13)
P. europea	Real time	*Ypt1*	Schena et al. (239)
P. fragariae	Conventional	ITS	Bonants et al. (35)
		ITS	Lacourt et al. (168)
		SCAR	Ioos et al. (135)
		ras	Ioos et al. (136)
		trp1	Ioos et al. (136)
	Real time	ITS	Bonants et al. (38)
P. fragariae rubi	Conventional	ITS	Schlenzig (242,243)
P. ilicis	Real time	*Ypt1*	Schena et al. (239)
P. infestans	LCR[b]	ITS	Tooley et al. (270)
	Conventional	ITS	Hussain et al. (129), Tooley et al. (267,269), Trout et al. (273)
		SCAR	Judelson and Tooley (146)
	Real time	*ras*	Atallah and Stevenson (13)

(*continued on next page*)

[a] Validation of species specificity was conducted at varying levels among these citations; it is advisable to confirm specificity prior to their use.
[b] LCR = ligase chain reaction; LAMP = loop mediated isothermal amplification.
[c] When tested against 13 *Pythium* spp., 5 of them produced a positive reaction.

regulated quarantine organism, from a broader perspective having a genus-specific marker capable of being multiplexed with a species-specific marker would enhance diagnostic capabilities by confirming if a *Phytophthora* spp. was responsible for the observed disease symptoms. A *Phytophthora* genus-specific diagnostic marker multiplexed with a species-specific marker was developed for conventional PCR based on the mitochondrially encoded *cox1-2* spacer region (198). One advantage of this marker system is that this region has been sequenced for all described species and the data posted on publicly available websites (www.phytophthoradb.org, www.phytophthora-id.org), so amplicons generated from environmental samples can be sequenced and identified by BLAST analysis (if multiple species are present cloning will be required). Bilodeau et al. (29) developed a multiplexed TaqMan real-time PCR assay for *P. ramorum* that included a genus-specific marker based on the β-tubulin gene, although some cross reactivity with other oomycetes was observed. Other genus-specific diagnostic markers have been reported for singleplex detection of *Phytophthora* spp. The ras-related *Ypt*-1 gene has several conserved exons separated by variable introns that exhibit enough variation to be useful for development of *Phytophthora* genus- and species-specific diagnostic markers that did not cross amplify the *Pythium* spp. tested (239). Several genus-specific markers based on the ITS region have been reported as well; Drenth et al. (71) described a conventional PCR primer pair that amplified a 752–832 bp amplicon that could be used with RFLP analysis for species identification, while Kox et al. (164) reported a genus-specific TaqMan real-time PCR marker, although some *Pythium* spp. were also detected with this latter marker. Having a high copy target sequence like the ITS or mitochondrial DNA will improve the sensitivity of the assay over single copy targets like *Ypt1* or β-tubulin. Even when using one of the higher copy regions, it may be necessary to do a nested amplification to visualize bands when doing conventional PCR with DNA extracted from plant tissue due to the low concentration of the pathogen relative to the plant DNA (198). In addition to genus-specific detection, diagnostic markers for amplification at a higher ordinal level have also been reported and may be useful as first round nested primers for detection from environmental samples (ITS [29,58]; 28S rRNA [12]; small rRNA subunit [23]; mitochondrially encoded cytochrome b region [93]).

Species-specific diagnostic markers. While random clones and cloned RAPD amplicons (Table 2) have been used for designing species-specific markers, a more efficient approach has been to sequence specific regions from a number of species and use unique regions to design species-specific primer pairs or TaqMan probes. Due to the large number of sequences available, this approach has historically focused on the ITS region, but more recent work has also looked at the ras-related protein *Ypt1* (238,239), β-tubulin and elicitin (29,31), and the spacer region between the mitochondrially encoded *cox1* and *cox2* genes (198,271). Multiplexing of assays developed from different loci improved the accuracy of results (29,194). The ITS, elicitin gene family, and *cox1* and *cox2* spacer region are higher copy number and hence may provide greater sensitivity than single copy regions such as the *Ypt1* and β-tubulin

Table 2. (*continued from preceding page*)

Species	Assay type	Locus	Reference
P. inundata	Real time	*Ypt1*	Schena et al. (239)
P. kernoviae	Real time	*Ypt1*	Schena et al. (239,240)
		ITS	Hughes et al. (125)
P. lateralis	Conventional	ITS	Winton and Hansen (295)
	Real time	*Ypt1*	Schena et al. (239)
P. medicaginis	Conventional	ITS	Liew et al. (180)
P. megasperma	Real time	*Ypt1*	Schena et al. (239)
P. melonis	Conventional	ITS	Wang et al. (284)
	Real-time	ITS	Wang et al. (284)
P. nemorosa	Real time	*Ypt1*	Schena et al. (239)
	Conventional	*cox* spacer	Martin et al. (198)
P. nicotianae	Conventional	SCAR	Ersek et al. (81), Goodwin et al. (96)
		elicitin	Lacourt and Duncan (169)
		ITS	Boersma et al. (33), Grote et al. (104), Huang et al. (123), Tooley et al. (267)
		parA1	Kong et al. (160)
		Ypt1	Meng and Wang (203)
	Real time	ITS	Huang et al. (123), Ippolito et al. (137), Schena et al. (241)
P. pinifolia	Conventional	ITS	Durán et al. (76)
	Conventional	*Ypt1*	Durán et al. (76)
P. pseudosyringae	Real time	*Ypt1*	Schena et al. (239)
	Conventional	*cox* spacer	Martin et al. (198)
	Real-time	*cox* spacer	Tooley et al. (271)
P. psychrophila	Real time	*Ypt1*	Schena et al. (239)
P. quercina	Conventional	SCAR	Nechwatal et al. (211), Schubert et al. (245)
	Real time	*Ypt1*	Schena et al. (239,240)
P. ramorum	Conventional	ITS	Garbelotto et al. (89)
		cox spacer	Martin et al. (198)
		SCAR	Ioos et al. (135)
		gpa1	Ioos et al. (136)
		trp1	Ioos et al. (136)
		cox1	Kroon et al. (167)
	Real-time	ITS	Bilodeau et al. (31), Hayden et al. (114,115), Hughes et al. (126), Tomlinson et al. (264)
		cox spacer	Tooley et al. (271)
		Ypt1	Schena et al. (239,240)
		elicitin	Bilodeau et al. (31)
		β-tubulin	Bilodeau et al. (31)
	LAMP[b]	ITS	Tomlinson et al. (264)
P. sojae	Real-time	ITS	Bienapfl et al. (27), Wang et al. (285)
Multiple spp.	PCR-ELISA	ITS	Bailey et al. (15)
Genus-specific	Conventional	ITS	Drenth et al. (71)
	Primers	*cox* spacer	Martin et al. (198)
	Real time	ITS[c]	Kox et al. (164)
		Ypt1	Schena et al. (239)

markers. The sequence alignments of these regions used for designing the species-specific markers, as well as additional background information on their use, may be found in the diagnostic section of the *Phytophthora* Database (www.phytophthoradb.org). A listing of the species for which diagnostic markers have been developed is presented in Table 2. Recently, a new diagnostic marker system has been developed for pathogen detection at a genus- and species-specific level using a multiplexed approach with a single pair of amplification primers and multiple TaqMan probes (G. Bilodeau, F. N. Martin, and M. D. Coffey, *unpublished*). The amplification primers span a region of gene order differences between *Phytophthora* compared to plants and *Pythium*, thereby enhancing the specificity of detection. Species-specific probes that anneal to the genus-specific amplicon have been validated for 14 species, and sequence analysis for over 500 isolates representing most described species in the genus indicates this locus should provide a systematic approach for marker development for most species.

Important concepts to keep in mind when using molecular diagnostic techniques. With the development of rapid and sensitive techniques such as real-time PCR and the availability of sequence data for a number of loci, the ability to develop a sensitive species-specific marker system is becoming less of a challenge. There are, however, additional concepts to note while using molecular diagnostic markers.

DNA extraction. The level of sensitivity and specificity of any DNA-based diagnostic assay is only as good as the starting extraction. Good quality, amplifiable DNA free of PCR inhibitors must be extracted from the environmental sample for these techniques to work properly. Instead of using traditional chloroform/phenol DNA extraction techniques, most diagnostic labs currently use commercial DNA extraction kits due to their ease of use, potential for high throughput sample processing, and lack of a need for hazardous waste disposal. Depending on the kit and sample material being extracted, different quantities of PCR inhibitors may make it through the extraction process; trying to include too much sample in the extraction will enhance this problem. One approach to reduce this problem is to dilute the DNA to reduce the concentration of inhibitors; however, this also dilutes the target DNA and may lower the sensitivity of the diagnostic assay. Additional purification steps using polyvinylpyrrolidone (164,174) or paramagnetic beads (28,164,177; P. Uribe and F. Martin, *unpublished*) have been used to improve the purity of the extracted DNA and thereby reduce the need for sample dilution. Bilodeau et al. (28) found that including an internal control in the amplification master mix significantly improved the ability to optimize the DNA extraction procedure from soil by providing a constant reference point for evaluating amplification efficiency.

Optimizing sensitivity of detection. As noted above, in addition to using DNA free of PCR inhibitors, selection of target sequences that are multiple copy is essential for obtaining a high level of diagnostic sensitivity. This is one advantage of the rDNA region, as it is present in much greater copy number than putative "single copy" genes such as β-tubulin. The selection of target sequences in other highly repetitive DNA can also increase the sensitivity of detection. Judelson and Tooley (146) obtained 100 times greater sensitivity targeting a highly repetitive element in *P. infestans* (14,000 copies per nucleus) compared to ITS detection. The mitochondrial DNA is also high in copy number relative to single copy nuclear genes. For TaqMan real-time PCR, the choice of master mixes can have an effect on sensitivity of detection; Bilodeau et al. (28) observed that for quantification of *V. dahliae,* the C_t was reduced by approximately 4 rounds when Real Master Mix without Rox (5 Prime, Gaithersburg, MD) was used rather than TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) for amplification of the same template DNA.

Multiplexing genus- and species-specific markers. While employing a species-specific diagnostic marker is essential for detecting the presence of a particular species, having the ability to determine if other *Phytophthora* spp. are present as well will enhance the information that can be obtained and perhaps identify new species. For example, including a genus-specific diagnostic marker in surveys of an ecosystem for a particular species will provide additional information on species communities, thereby allowing a broader view of the impact of *Phytophthora*. The availability of a sequence database for the amplified region broadly reflecting the genus will facilitate identification of what is present to a species level by BLAST analysis. If two species are present in a sample, cloning of the amplicon will be necessary in order to get clean sequence data.

Including positive or internal controls. To avoid false negatives due to the presence of PCR inhibitors preventing amplification, a positive control that will amplify in all samples should be multiplexed with the diagnostic marker. Target regions for these markers found in environmental samples that have been used include *cox1* gene for plants (126,198,271), the 18S rDNA region (240,295), and RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, 29). Judelson and Tooley (146) developed a positive control for their assay of *P. infestans* by cloning a band generated from *E. coli* by amplification with pathogen-specific primers at reduced stringency. In cases where pathogen quantification is desired, it is important to include an internal control so the presence of PCR inhibitors that may be reducing the amplification efficiency of the diagnostic marker (and hence increasing the C_t) can be identified. This can be done by adding a specific amount of internal control DNA to the amplification master mix and evaluating amplification compared to a water control amplification. Ideally, the amplification primers for the internal control would be the same as the pathogen diagnostic amplicon, but the TaqMan probe would be specific for the internal control. While this type of internal control has not been described for diagnostics of *Phytophthora* spp., it has for *Phakopsora pachyrhizi* (113) and *Verticillium dahliae* (28), and they should be amenable for modification for use with *Phytophthora*. When using positive and internal controls, it is important to determine if multiplexing reduces the amplification efficiency of the pathogen target amplicon.

Pathogen quantification. In addition to using an internal control in the assays used to quantify the amount of the pathogen, it is also important that the target sequence be present in a relatively constant amount among all isolates. While the ITS region of the rDNA has been the target sequence for a number of species-specific diagnostic assays, intraspecific differences in rDNA copy number have been reported for a range of Eumycotan fungi (48,185,223, 227,234). Although there is no direct evidence to confirm that this occurs in *Phytophthora*, there is indirect evidence suggesting that it does occur in the genus *Pythium* (192) and with *Phytopythium vexans* (252) and hence should be considered when designing quantification assays for *Phytophthora*. While rDNA copy number in isolates of *Verticillium dahliae* was recently reported to vary between approximately 24 and 73 copies depending on the isolate, the impact on the results of soil quantification was estimated to be a maximum difference of 1.8 C_t; based on regression analysis between C_t and soil plate counts of inoculum density ($R^2 = 0.96$), this potential difference in copy number among isolates did not appear to influence the accuracy of results obtained for field soils collected from different regions of California (28).

Marker validation. Adequate validation of diagnostic markers is essential. This includes testing against isolates of the target pathogen collected from diverse regions to ensure a high degree of intraspecific conservation of annealing sites, evaluation of geographically diverse isolates of phylogenetically closely related species that have a higher degree of homology in annealing sites to ensure species specificity, a broad representation of species in the genus, and other microorganisms present in the samples that will be processed. At some point in this evaluation of specificity, DNA from the plants that will be assayed should be added to the master mix at a similar concentration to that used when assaying field samples to ensure there is no inhibition of target amplification (there have been examples where pathogen primers have annealed to host DNA [245]). Finally, marker validation should be done with envi-

ronmental samples when culturing or DNA sequence analysis of amplicons has been done to confirm the presence of particular species.

Considerations when evaluating markers developed in another lab. When first evaluating diagnostic markers reported in the literature, it is important to follow the amplification described procedure right down to the brand of *Taq* polymerase that was used. In the Martin lab we have noticed major differences in specificity and sensitivity of some TaqMan real-time PCR assays when the brand of master mix was changed from one company to another. This may also be possible when doing conventional PCR amplifications. Since thermal cyclers may be calibrated differently, we often run the initial amplification with an annealing temperature 2°C lower than recommended and then increase this to obtain specificity.

Phytophthora Database

In an effort to provide a comprehensive repository for information on the genus *Phytophthora*, a web portal called the *Phytophthora* Database (www.phytophthoradb.org) was developed (222). This database includes a listing of the species, their morphological features (for some species a listing of their hosts and pictures of diseased plants are presented), geographic distribution, and a listing of references about the genus. One important function of the project was to provide a comprehensive phylogenetic framework for the genus and tools that could be used for molecular identification. Eight nuclear and six mitochondrial loci have been sequenced for representative isolates of each species with the nuclear phylogeny published by Blair et al. (32) presented at the top of each species page. In the "Genetic Marker" section, there is a listing of primers and amplification conditions for all loci that have been sequenced. As of July 2011, a total of 6,192 sequences from 2,593 isolates representing 107 species were posted and are available for BLAST analysis for identification of unknown isolates. It is also possible to download the sequences as well as conduct some forms of analysis. Many of the cultures used to generate the sequence data came from the World Oomycete Genetic Resource Collection at the University of California, Riverside, and are available for other researchers to request. To reduce the need for obtaining permits for movement of pathogens, a bank of DNA extracted from a number of isolates has been established and can be obtained through the website (http://phytophthora.ucr.edu/WPCcharges.htm). There is also a section on techniques for molecular identification and diagnostics that includes the sequence alignments used in the development of several genus- and species-specific marker systems to facilitate development of markers for additional species. While registration and log-in are not required to use the database, logged in users are able to store some of their analysis on the database server for future work. This website is a work in progress, and suggestions for improvement and contribution of content by the research community is encouraged.

Future Directions of Research

In the past decade, there have been significant advances in the molecular methods used for isolate identification, monitoring of populations, and species-specific detection. Several areas where further advancements would improve the capability of the research and regulatory community in these areas include:

- Having a comprehensive resource of information on the types (holotype, ex-holotype, neotype, and ex-neotype) would facilitate species identification as well as description of new species. Along these lines, work is in progress on completion of "Morphological/Molecular Identification Tools for *Phytophthora* based on the Types" that contains a Lucid Key, Tabular Key (expansion of Table 1), information on the types in different culture collections around the world (including the various accession numbers used for them), and datasheets all based on the morphological and molecular characters of the types and selected neotypes candidates (Z. G. Abad, Y. Balci, M. Coffey, and S. Kang, *unpublished*). It is also important to note the plasticity in morphological as well as physiological features when describing a novel species, which becomes evident only when a diverse set of isolates that represent the population is examined. Thus, novel species descriptions should include a variety of isolates to reduce confusion in species identification as well as DNA sequence data from a minimum of several loci to confirm uniqueness. In an effort to reduce confusion and make information on newly discovered species available to the research community, it also is important to finish formal species descriptions instead of relying on provisional species designations.
- Development of techniques that will reduce the need for specialized equipment or time that it takes to process a sample. The development of procedures where the nitrocellulose membrane from a serological detection kit is used as a source of DNA (eliminating the need for doing a DNA extraction procedure) and isothermal amplification is used for pathogen detection (265) is a step in the right direction. Additional testing is needed to see if this approach works for a wider range of host tissue and pathogen diagnostic markers. Another approach for isothermal amplification under commercial development (EnviroLogix, Portland, ME) is nicking enzyme amplification reaction (NEAR [251]). This approach utilizes enzymes capable of nicking double-stranded DNA at specific locations, a strand displacing DNA polymerase, and a set of primers with nicking enzyme binding sites in the 5′ end to conduct isothermal amplification. It has been reported to be influenced less by contaminants that make it through the DNA extraction procedures than PCR, and while it is currently being tested for detection of bacterial pathogens, it may also be useful for *Phytophthora* spp. While the "lab on a chip" described by Julich et al. (147) is technologically more complex than current techniques, it does provide a rapid (as short as 10 minutes) and sensitive means for detecting specific *Phytophthora* spp.
- Development of techniques to assess pathogen viability. While DNA from nonviable cells will degrade over time in the soil or plant tissue, the rate of degradation will vary depending on a number of factors, including environmental conditions. Having the ability to differentiate if the results of a diagnostic assay were from viable or nonviable pathogen cells would be helpful when making regulatory decisions. One technique for accomplishing this is to use an RNA template and reverse transcription prior to the PCR assay, an approach that has been used for *P. cambivora* (282) and *P. ramorum* (54) with *cox1* or *cox2* genes. Additional work in this area is needed to determine if this approach will work for other species as well.
- Improvement in approaches for determining cut-off values for positive results when running real-time PCR. This has been approached by using a C_t value of 40, determining at what value the regression analysis between pathogen concentration and C_t is no longer linear, or determining the point where false positives are observed when a specific concentration of DNA from a related species is used. Ideally, the cut-off value would be determined by evaluating the presence or absence of the pathogen from a large dataset using multiple techniques to confirm results and be statistically supported using an approach similar to what was reported by Chandelier et al. (53) for a *P. ramorum* real-time PCR detection technique.
- Differentiating false positive results from valid positives. Differences in amplification kinetics between false positive and true positive amplifications can be observed in some real-time PCR diagnostic assays. Modeling a large enough sample size of these amplifications to identify specific characteristics of a false positive amplification may provide insight on additional techniques for improving the accuracy of pathogen detection. Sigmoidal curve fitting analysis has been used to model amplification kinetics to improve the accuracy of real time PCR quantification assays (235) and should be useful for this purpose as well.
- Development of primers specific for amplification of different genera or families of oomycetes that could be used in community analysis. As the results of Arcate et al. (12) suggested

using an oomycete specific primer based on the 28S rDNA, there are likely to be oomycetes in the soil ecosystem that have yet to be described. Bent et al. (23) designed a straminipile specific primer pair from the 18S region of the rDNA that will be useful for this purpose as well. Having a better selection of amplification primers to use would improve our ability to identify these and enhance our understanding of the diversity of oomycetes in the ecosystem. Knowing if there is variation in the copy number of the target sequences for different genera or species would be helpful for developing quantification assays.

- Our knowledge of diversity of the genus *Phytophthora* is largely based on observations when they were found in association with a plant disease; however, the widespread occurrence of diverse newly described *Phytophthora* species in forest ecosystems suggests that a broader ecosystems approach to analysis is needed. A systematic survey approach that includes stream, soil, and aboveground plant parts is needed to reveal the true diversity that exists within this genus and will help to fill in the taxonomic gaps. Furthermore, the biodiversity of *Phytophthora* spp. in natural ecosystems is largely based on temperate forests, leaving out the most diverse environment, the tropics. It is possible that some of the recent emerging temperate forest pathogens may have originated from increasingly disturbed tropical or subtropical environments.
- The ecological roles of many *Phytophthora* species remain enigmatic, particularly for species routinely found in streams. These appear to be part of the saprotrophic mycobiota similar to what has been observed for *Halophytophthora*, a sister genus commonly found in brackish water. A microbial approach that takes into account their functional roles in ecosystem health and their influence on plant diversity (by creating a negative feedback) may change our view on functional roles of this group of organisms.

Conclusion

The past decade has not only seen an approximate doubling in the number of described species within the genus *Phytophthora*, but a significant increase in the availability of DNA sequence data covering a variety of loci deposited in public databases. This has improved our ability to identify isolates to a species level as well as provided a phylogenetic framework for description of new species and better resolution of species complexes. Further collection of sequence data from geographically diverse isolates representing the range of variation within a species will provide the necessary data to delineate species boundaries and clarify the species concept in the genus. While this work is improving our ability to identify species based on phylogenetic grouping, it has also revealed that the genus has a much greater diversity than previously appreciated. With increased sampling of nonagricultural ecosystems, it is likely the list of new species will continue to grow, highlighting the need for a more thorough taxonomic evaluation of the genus that integrates both morphological and molecular criteria as well as a reevaluation of the ecological impact of this important genus.

The DNA sequences that have been collected for phylogenetic and identification purposes have also provided a resource that is useful for developing diagnostic assays. Genomic sequencing of several species and related genera should help in identifying additional loci that will be useful for designing hypervariable markers for population studies as well as loci that are stable on an intraspecific level that will be useful for developing species-specific diagnostic markers. While the development and widespread adoption of real-time PCR has revolutionized molecular diagnostics, the technology has not reached its full potential outside of a research setting due to factors such as cost of consumable supplies, the need to purchase specialized equipment, and the level of technical expertise needed to perform the assays. Further development of techniques for obtaining pathogen DNA amenable for high throughput processing that will provide clean template DNA, as well as developing assay techniques requiring less specialized equipment and technical expertise (such as isothermal amplification), will enhance our opportunities for a more widespread adoption of these technologies.

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