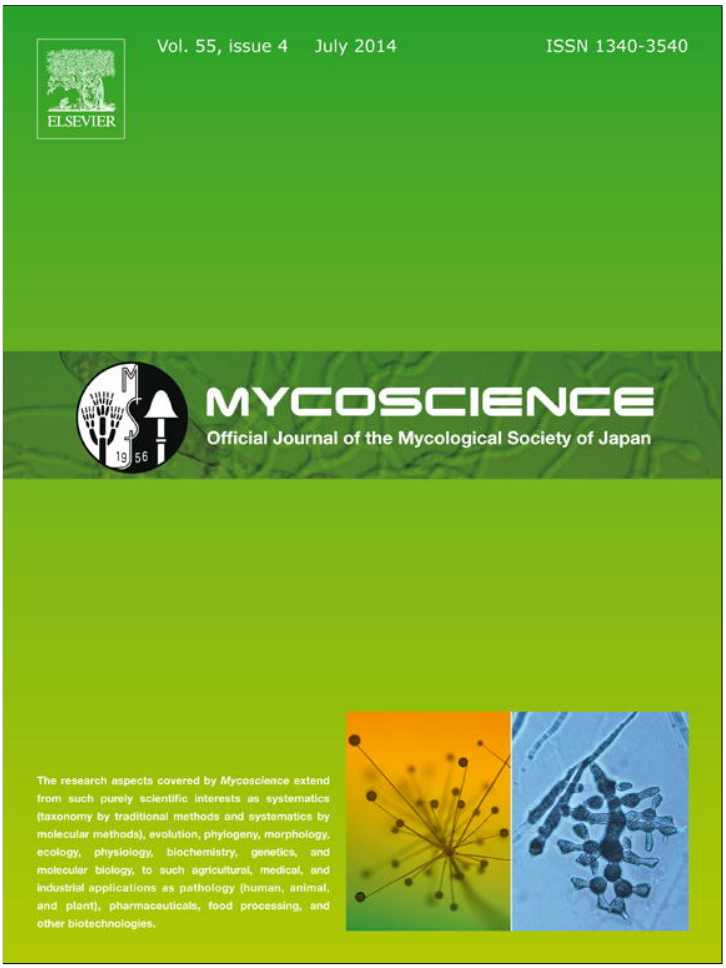

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Full paper

Re-evaluation of Japanese *Phytophthora* isolates based on molecular phylogenetic analyses

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ABSTRACT

Over the past 40 years in Japan, *Phytophthora* isolates have been collected from various diseased host tissues and infested soils and identified using morphological characters. In order to develop a molecular method for the characterization of Japanese *Phytophthora* species, we obtained nuclear ribosomal ITS and LSU and mitochondrial *cox*I DNA sequences from 151 isolates representing 21 known species and 10 unidentified isolates. These were compared with similar sequences from representative isolates of known species. Of these, 124 isolates were found to have been correctly identified. Among the remaining 37 isolates, 19 showed high homology with other described species. The remaining 18 isolates showed only low levels of homology with any known species, and generated monophyletic sub-clades in a phylogenetic tree based on the ITS and nLSU regions and the *cox*I gene. Therefore, these isolates are candidates for new species, falling into six groups. Together, the Japanese isolates were found to represent phylogenetically diverse groups of species. In a sequence variation analysis, the ITS regions and the *cox*I genes were found to be more variable than the nLSU sequences, suggesting that they will be more useful for *Phytophthora* identification.

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1. Introduction

The genus *Phytophthora* includes a diverse group of plant pathogens that infect a broad range of hosts in both agricultural and natural environments all over the world, including Japan (Erwin and Ribeiro 1996; Blair et al. 2008). Until the mid 1990s only 54 *Phytophthora* species had been described worldwide (Erwin and Ribeiro 1996). Currently, the genus consists of about 120 species (Kroon et al. 2012). *Phytophthora* species produce zoospores under favorable conditions, and these help the pathogens to spread, with devastating consequences. Moreover, since hydroponic plant culture has become popular in Japan, *Phytophthora* species are causing serious losses because they can spread easily through water. The rapid and accurate diagnosis of isolates is crucial for the proper control of *Phytophthora* diseases.

Proper identification to the species level is a critical first step in the investigation of any pathogen. However, the traditional taxonomy of *Phytophthora* species is based on morphological characteristics (Waterhouse 1963) and does not reflect natural phylogenic relationships (Cooke et al. 2000; Kroon et al. 2004). Difficulties in morphological analyses have led to misidentifications. Recent molecular techniques provide greatly enhanced approaches for the identification and circumscription of species. The most common genomic regions used for identification of Oomycetes to the species level is the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA (rRNA) gene. This region is non-coding; it evolves rapidly, and is highly variable. Universal primers can be used to amplify the ITS regions of any *Phytophthora* isolate prior to sequencing (Ristaino et al. 1998). Cooke et al. (2000) first used the ITS regions in a phylogenic study of all known *Phytophthora* species, and since then, this region has been used extensively in phylogenic studies of Oomycetes.

Some coding regions, including the large sub-unit of nuclear rRNA gene (nLSU) and the genes encoding the cytochrome *c* oxidase subunits I and II (*cox*I and *cox*II), are also being using in phylogenic studies of *Phytophthora* (Martin and Tooley 2003). Blair et al. (2008) used seven nuclear and mitochondrial loci to obtain a comparatively good resolution of the species relationships. The *cox*I gene has recently received attention because it is mitochondrially encoded, and alignments are simple and devoid of gaps if introns are absent. Results from studies of insects and *Penicillium* suggested that sequence alignments of the *cox*I gene are more reliable than those of rRNA genes for accurate species delimitations (Hajibabaei et al. 2006; Seifert et al. 2007). Martin and Tooley (2003) and Kroon et al. (2004) used *cox*I in their phylogenetic studies of oomycete genera. Recently, Robideau et al. (2011) extended this work by sequencing the *cox*I gene from 1205 isolates of 23 oomycete genera. In some cases, the *cox*I gene was more discriminative than the ITS regions at the species level. Thus the "*cox*I barcode" may be an excellent tool for species identification (Ekrem et al. 2007). Furthermore, since both the *cox*I gene and the ITS regions each provide acceptable resolution (Bala et al. 2010), a barcode using both sequences may be even more useful for species identification in the Oomycetes (Robideau et al. 2011).

There have been few molecular phylogenetic analyses of *Phytophthora* species in Japan, and there is some concern that errors have occurred in species identification in this country. Villa et al. (2006) suspected that a Japanese isolate assigned to *P. sojae* Kaufmann & Gerdemann might be a misidentification of *P. citricola* Sawada. Similarly, a Japanese isolate of *P. citricola* was identified as *P. gregata* T. Jung, Stukely & T. I. Burgess (Jung et al. 2011). Uddin et al. (2007) studied the phylogenetic relationships among Japanese isolates of *P. citrophthora* (R. E. Smith & E. H. Smith) Leonian and found some intra-isolate variation. Moreover, the number of described *Phytophthora* species has increased, as mentioned above. Therefore, there is a need to re-check the Japanese isolates and perform a phylogenetic analysis using DNA sequences. An analysis of inter-specific and intra-specific variations in the sequences is needed to clarify the boundaries between species. The objective of this study was to re-examine a collection of Japanese isolates of the genus *Phytophthora* by performing a homology search and a phylogenetic analysis using sequences from the ITS regions, the nLSU, and the *cox*I gene.

2. Materials and methods

2.1. Isolates

In this study we examined 161, Japanese isolates of *Phytophthora* (Table 1), of which 105 were our own isolates, which originally identified based on morphological observations and mycelial growth, 44 were collected from Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan and 12 from NITE Biological Resource Centre (NBRC), Japan. Of these, 151 isolates had been identified at the species level and represented 21 of the 31 species known to occur in Japan, while the remaining 10 isolates were unidentified *Phytophthora* species. All isolates were maintained on corn meal agar (CMA) (to 20 g of corn meal added 1 l of water, steamed for 30 min, filter the extract and ensure the volume 1 l and add 20 g agar, autoclave for 1 h at 121 °C) in the Gifu University culture collection.

2.2. Phylogenetic analysis

2.2.1. DNA extraction

Isolates were grown for 7 d in V8A medium (100 ml V-8 juice, 2.5 g $CaCO_3$, 20 g agar and 900 ml distilled water). Most were grown at 25 °C, while some were grown at other temperatures (between 20 °C and 25 °C) according to their requirements. For each DNA sample, a small amount of mycelium mat from an advanced growth area was collected in 100 μl of 50% PrepMan Ultra Reagent (Applied Biosystems, Foster city, CA, USA) and heated to 100 °C for 10 min. After 3 min incubation at room temperature, the sample was centrifuged at 15,000 rpm for 3 min. The supernatant was transferred to another tube with 100 μl TE buffer (10 mM Tris–HCl, pH 7.5 and 0.1 mM EDTA), and this was used for PCR.

2.2.2. DNA amplification

The ITS regions, nLSU, and *cox*I were amplified using the primer sets shown in Table 2. We used 25 μl reaction mixtures containing 1 μl DNA, 0.2 μM of each primer (for ITS and nLSU)

Table 1 – List of *Phytophthora* isolates used in this study, including clade position, location, year of collection, host, and accession numbers.

Species	Clade no.[a]	Isolate[b]	Location in Japan	Year of collection	Host	GenBank accession no		
						ITS	nLSU	coxI
P. cactorum	1a	CH 98LOQ1	Chiba	1998	Loquat	AB367362	AB688426	AB688152
P. cactorum	1a	CH 88-26	Chiba	1988	Loquat	AB367363	AB688427	AB688153
P. cactorum	1a	CH 98PEC1	Chiba	1998	Japanese pear	AB367364	AB688428	AB688154
P. cactorum	1a	CH 02MKPY001	Tokushima	2002	*Aralia elata*	AB367365	AB688429	AB688155
P. cactorum	1a	CH 03OKType1	Okayama	2002	Strawberry	AB367366	AB688430	AB688156
P. cactorum	1a	CH 99LU1	Chiba	1999	Russell lupin	AB367367	AB688431	AB688157
P. cactorum	1a	GF654	Gifu	2004	Strawberry	AB688317	AB688432	AB688158
P. cactorum	1a	MAFF 235099	Niigata	1985	Tulip	AB688318	AB688433	AB688159
P. cactorum	1a	CH 98PA11	Hokkaido	1998	*Paeonia albiflora*	AB688319	AB688434	AB688160
P. cactorum	1a	NBRC 32194	Niigata	1985	Tulip	AB217672	AB688435	AB688161
P. cactorum	1a	NBRC 30474	Hokkaido	1976	Soil of apple orchard	AB688320	AB688436	AB688162
P. cactorum	1a	MAFF 731066	Iwate	1998	Strawberry	AB688321	AB688437	AB688163
P. cactorum	1a	MAFF 103068	Tochigi	1987	Strawberry	AB688322	AB688438	AB688164
P. cactorum	1a	MAFF 240271	Tokushima	2002	Angelica	AB688323	AB688439	AB688165
P. cactorum	1a	MAFF 305800	Tokyo	1988	*Aralia cordata*	AB688324	AB688440	AB688166
P. cambivora	7a	MAFF 305918	Hokkaido	1987	Apple	AB367381	AB688441	AB688167
P. capsici	2b	CH 99PHM1	Chiba	1999	*Cucumis melo*	AB367368	AB688442	AB688168
P. capsici	2b	CH 92MW2	Chiba	1992	Watermelon	AB367369	AB688443	AB688169
P. capsici	2b	CH 02K0104-1	Chiba	2002	*Cucurbita maxima*	AB367370	AB688444	AB688170
P. capsici	2b	CH 01CUCU10	Saitama	2001	Cucumber	AB367371	AB688445	AB688171
P. capsici	2b	CH 02UE0202	Nagano	2002	Sweet pepper	AB688325	AB688446	AB688172
P. capsici	2b	NBRC 30696	Hokkaido	–	*Cucurbita* sp.	AB217670	AB688447	AB688173
P. capsici	2b	MAFF 305920	Shizuoka	1981	Water melon	AB688326	AB688448	AB688174
P. capsici	2b	MAFF 305921	Shizuoka	–	Egg plant	AB688327	AB688449	AB688175
P. capsici	2b	MAFF 305957	Okayama	1988	Tomato	AB688328	AB688450	AB688176
P. capsici	2b	NBRC 8386	Kyoto	–	–	AB688329	AB688451	AB688177
P. capsici	2b	NBRC 30697	–	–	*Cucurbita maxima*	AB688330	AB688452	AB688178
P. cinnamomi	7b	CH 95PHE1	Ibaraki	1995	*Eustoma grandiflorum*	AB367374	AB688453	AB688179
P. cinnamomi	7b	CH00Hyp4	Kochi	2000	*Hypericum androsaemum*	AB367375	AB688454	AB688180
P. cinnamomi	7b	NBRC 33182	Kochi	2000	*Hypericum androsaemum*	AB217675	AB688455	AB688181
P. cinnamomi	7b	C34	Chiba	2007	*Limonium sinuatum*	AB688331	AB688456	AB688182
P. cinnamomi	7b	MAFF 238144	Kochi	1999	*Hypericum androsaemum*	AB688332	AB688457	AB688183
P. cinnamomi	7b	CT09CHAP1	Chiba	2009	*Chamaecyparis obtusa*	AB688333	AB688458	AB688184
P. cinnamomi	7b	NBRC 33180	Kochi	1999	*Hypericum androsaemum*	AB688334	AB688459	AB688185
P. citricola	2	CH 95PHE28	Chiba	1995	*Eustoma grandiflorum*	AB367376	AB688460	AB688186
P. citricola	6	CH 97TUL2	Chiba	1997	*Tulipa gesneriana*	AB367377	AB688461	AB688187
P. citricola	2	CH 98U121C	Chiba	1998	*Citrus unshiu*	AB367378	AB700470	AB688188
P. citrophthora	2a	CH 95PHE29	Chiba	1995	*Eustoma grandiflorum*	AB366366	AB688462	AB688189
P. citrophthora	2a	CH 94HE11	Tokyo	1994	*Hedera canariensis*	AB366367	AB688463	AB688190
P. citrophthora	2a	CH 90-15	Chiba	1990	Kiwi fruit	AB366370	AB688464	AB688191
P. citrophthora	2a	CH 98U6B	Chiba	1998	*Citrus unshiu*	AB366376	AB688465	AB688192
P. citrophthora	2a	CH 04U1B	Chiba	2004	*Citrus unshiu*	AB366380	AB688466	AB688193
P. citrophthora	2a	CH 92IC1	Chiba	1992	*Ficus carica*	AB366384	AB688467	AB688194
P. citrophthora	2a	CH 88IC1	Chiba	1988	*Ficus carica*	AB769172	AB688468	AB688195
P. colocasiae	2a	NBRC 30695	Chiba	–	*Colocasia antiquorum*	AB688335	AB688469	AB688196
P. cryptogea	8a	C27	Chiba	2007	Carnation	AB688336	AB688470	AB688197
P. cryptogea	8a	C30	Chiba	2007	Sunflower	AB688337	AB688471	AB688198
P. cryptogea	8a	C37	Tokyo	2007	*Tradescantia spathacea*	AB688338	AB688472	AB688199
P. cryptogea	8a	MAFF 306438	Chiba	1992	*Matthiola incana*	AB366729	AB688473	AB688200
P. cryptogea	8a	CH 92-12	Chiba	1992	*Solanum mammosum*	AB366731	AB688474	AB688201
P. cryptogea	8a	MAFF 306433	Chiba	1990	*Ammi majus*	AB366735	AB688475	AB688202
P. cryptogea	8a	CH 95PHE26	Chiba	1995	*Eustoma grandiflorum*	AB366737	AB688476	AB688203
P. cryptogea	8a	CH 95PHE10	Ibaraki	1995	*Eustoma grandiflorum*	AB366739	AB688477	AB688204
P. cryptogea	8a	MAFF 306431	Chiba	1985	Gerbera	AB366742	AB688478	AB688205
P. cryptogea	8a	CH 85-21	Chiba	1985	Gerbeara	AB366743	AB688479	AB688206
P. cryptogea	8a	CH 95PHG1	Chiba	1995	Gerbera	AB366744	AB688480	AB688207
P. cryptogea	8a	MAFF 306439	Chiba	1992	*Solanum mammosum*	AB688339	AB688481	AB688208
P. cryptogea	8a	MAFF 306443	Chiba	1992	Pot marigold	AB688340	AB688482	AB688209
P. cryptogea	8a	MAFF 306694	Miyagi	2005	Gerbera	AB688341	AB688483	AB688210
P. cryptogea	8a	NBRC 32325	Kanagawa	1984	Gerbera	AB688342	AB688484	AB688211
P. chrysanthemi	10	MAFF 712282	Toyama	1998	*Chrysanthemum* sp.	AB688343	AB688485	AB688212
P. drechsleri	7b	CH 96HE1	Chiba	1996	*Hedera helix*	AB367382	AB688486	AB688213

Table 1 – (*continued*)

Species	Clade no.[a]	Isolate[b]	Location in Japan	Year of collection	Host	GenBank accession no		
						ITS	nLSU	*cox*I
P. drechsleri	7b	CH 96HE2	Chiba	1996	*Hedera helix*	AB688344	AB688487	AB688214
P. drechsleri	8a	C21	Chiba	2007	China aster	AB688345	AB688488	AB688215
P. drechsleri	8a	C32	Oita	2007	*Heteropappus hispidus*	AB688346	AB688489	AB688216
P. drechsleri	8a	MAFF 240294	Tokushima	2005	Indian spinach	AB688347	AB688490	AB688217
P. infestans	1c	MAFF 236324	Ibaraki	1992	Tomato	AB688348	AB688492	AB688219
P. infestans	1c	MAFF 305586	Hokkaido	1987	Potato	AB688350	AB688493	AB688220
P. megasperma	8a	CH 95PHG7	Chiba	1995	Gerbera	AB688351	AB688494	AB688221
P. megasperma	8a	CH 95PHG8	Chiba	1995	Gerbera	AB688352	AB688495	AB688222
P. megasperma	7b	Pm-1	Hokkaido	1997	*Glycine max*	AB688353	AB688496	AB688223
P. megasperma	6	MAFF 237500	Kagoshima	1987	*Lilium longiflorum*	AB688354	AB688497	AB688224
P. megasperma	7	CH 04PHR11	Chiba	2004	Rose	AB688355	AB688498	AB688225
P. megasperma	7	CH 04PHR12	Chiba	2004	Rose	AB688356	AB688499	AB688226
P. megasperma	7	CH 94PHR1	Chiba	1994	Rose	AB688357	AB688500	AB688227
P. megasperma	7	CH 95PHR10	Chiba	1995	Rose	AB688358	AB688501	AB688228
P. megasperma	7	CH 95PHR16	Chiba	1995	Rose	AB688359	AB688502	AB688229
P. megasperma	7	CH 95PHR17	Chiba	1995	Rose	AB688360	AB688503	AB688230
P. megasperma	7	CH 00MKR1	Chiba	2000	Rose	AB688361	AB688504	AB688231
P. megasperma	7	CH 00MKR2	Chiba	2000	Rose	AB688362	AB688505	AB688232
P. megasperma	7	CH 02PHR1	Kanagawa	2001	Rose	AB688363	AB688506	AB688233
P. megasperma	7	CH 97PHR1	Shizuoka	1997	Rose	AB688364	AB688507	AB688234
P. megasperma	7	P-A	Chiba	1968	Rose	AB688365	AB688508	AB688235
P. megasperma	7	P-B	Chiba	1974	Rose	AB688366	AB688509	AB688236
P. megasperma	8a	NBRC 31624	Hokkaido	–	Soil	AB217680	AB688510	AB688237
P. megasperma	6	NBRC 32176	Kagoshima	1988	*Lilium longiflorum*	AB217681	AB688511	AB688238
P. megasperma	8a	GF433	Gifu	2002	Gerbera	AB700474	AB700475	AB700476
P. megasperma	8a	GF543	Gifu	2003	Gerbera	AB688367	AB688512	AB688239
P. megasperma	8a	GF649	Gifu	2004	Gerbera	AB688368	AB688513	AB688240
P. melonis	7b	CH 00ME1-1	Kumamoto	2000	*Cucumis melo*	AB366545	AB688514	AB688241
P. melonis	7b	CH 00ME21-21	Miyazaki	2000	*Cucumis melo*	AB366549	AB688515	AB688242
P. melonis	7b	CH 94CUCU1	Fukushima	–	*Cucumis sativus*	AB366552	AB688516	AB688243
P. nicotianae	1	MAFF 305941	Chiba	1985	*Petroselinum sativum*	AB769174	AB688517	AB688244
P. nicotianae	1	CH 91-29	Chiba	1991	*Limonium* sp.	AB688369	AB688518	AB688245
P. nicotianae	1	MAFF 235793	Chiba	1987	*Dianthus* sp.	AB688370	AB688519	AB688246
P. nicotianae	1	CH 87WG1	Chiba	1987	*Dianthus caryophyllus*	AB688371	AB688520	AB688247
P. nicotianae	1	CH 91LK4	Chiba	1991	*Lilium longiflorum*	AB688372	AB688521	AB688248
P. nicotianae	1	MAFF 235785	Chiba	1989	*Bougainvillea* sp.	AB688373	AB688522	AB688249
P. nicotianae	1	CH 90-3	Chiba	1990	*Aphelandra squarrosa*	AB688374	AB688523	AB688250
P. nicotianae	1	CH 92ALS11	Chiba	1992	Alstoroemeria	AB367348	AB688524	AB688251
P. nicotianae	1	CH 92ORN11	Chiba	1992	*Ornithogalum* sp.	AB367352	AB688525	AB688252
P. nicotianae	1	CH 91-1	Chiba	1991	*Strelitzia reginae*	AB367344	AB688526	AB688253
P. nicotianae	1	CH 94AROE1	Chiba	1994	Aroe	AB688375	AB688527	AB688254
P. nicotianae	1	MAFF 410750	Chiba	1995	*Daphne odora*	AB688376	AB688528	AB688255
P. nicotianae	1	CH 97HE11	Chiba	1996	*Hedera helix*	AB688377	AB688529	AB688256
P. nicotianae	1	CH 00POIN2	Hyogo	2000	Poinsettia	AB688378	AB688530	AB688257
P. nicotianae	1	CH 94TK1	Chiba	1994	Tobacco	AB688379	AB688531	AB688258
P. nicotianae	1	CH 93ANE1	Chiba	1993	Anemone	AB688380	AB688532	AB688259
P. nicotianae	1	CH 98U1A	Chiba	1998	*Citrus unshiu*	AB367355	AB688533	AB688260
P. nicotianae	1	CH 98Y1A	Chiba	1998	*Citrus junos*	AB367356	AB688534	AB688261
P. nicotianae	1	MAFF 238154	Kochi	1999	*Allium fistulosum*	AB688381	AB688535	AB688262
P. nicotianae	1	T1234	Toyama	2000	*Allium fistulosum*	AB688382	AB688536	AB688263
P. nicotianae	1	MAFF 235784	Chiba	1989	*Vanda* sp.	AB688383	AB688537	AB688264
P. nicotianae	1	GF101	Gifu	–	Karankoe	AB688384	AB688538	AB688265
P. nicotianae	1	C01	Chiba	2006	*Gazania* sp.	AB688385	AB688539	AB688266
P. nicotianae	1	C08	Chiba	2006	*Ardisia crispa*	AB688386	AB688540	AB688267
P. nicotianae	1	C15	Tateyama	2006	*Echium fastuosum*	AB688387	AB688541	AB688268
P. nicotianae	1	F03	Fukuoka	2006	Southern cross	AB688388	AB688542	AB688269
P. nicotianae	1	C24	Tateyama	2007	*Abutilon* sp.	AB688389	AB688543	AB688270
P. nicotianae	1	C38	Chiba	2007	Southern cross	AB688390	AB688544	AB688271
P. nicotianae	1	MAFF 235436	Ibaraki	1983	*Daphne odora*	AB688391	AB688545	AB688272
P. nicotianae	1	MAFF 235590	Hokkaido	1982	*Solanum melongena*	AB688392	AB688546	AB688273
P. nicotianae	1	MAFF 238148	Kochi	1999	Chinese leek	AB688393	AB688547	AB688274
P. nicotianae	1	MAFF 238150	Chiba	1999	Glory lily	AB688394	AB688548	AB688275

(*continued on next page*)

Table 1 – (*continued*)

Species	Clade no.[a]	Isolate[b]	Location in Japan	Year of collection	Host	GenBank accession no		
						ITS	nLSU	coxI
P. palmivora	4	MAFF 235787	Chiba	1988	*Oncidium* sp.	AB688395	AB688549	AB688276
P. palmivora	4	CH 88-5	Chiba	1988	*Oncidium* sp.	AB367357	AB688550	AB688277
P. palmivora	4	CH 91-23	Tokyo	1991	*Strelitzia reginae*	AB688396	AB688551	AB688278
P. palmivora	4	CH 91-25	Tokyo	1991	*Strelitzia reginae*	AB 688397	AB688552	AB688279
P. palmivora	4	MAFF 235783	Chiba	1989	*Vanda* sp.	AB769175	AB688553	AB688280
P. palmivora	4	CH 89-38	Chiba	1989	*Vanda* sp.	AB367358	AB688554	AB688281
P. palmivora	4	CH 01Cat11	Chiba	2001	*Cattleya* sp.	AB367359	AB688555	AB688282
P. palmivora	4	CH 96Cymbi1	Chiba	1996	*Cymbidium* sp.	AB367360	AB688556	AB688283
P. palmivora	4	CH 96Cymbi2	Chiba	1996	*Cymbidium* sp.	AB688398	AB688557	AB688284
P. palmivora	4	CH 97Denpha1	Shizuoka	1997	Cooktown orchid	AB367361	AB688558	AB688285
P. palmivora	4	CH 97Denpha2	Shizuoka	1997	Cooktown orchid	AB688399	AB688559	AB688286
P. palmivora	4	S08	Shizuoka	2007	*Dinema poriburubon*	AB688401	AB688561	AB688288
P. palmivora	4	C82[c]	Okinawa	2007	*Strelitzia reginae*	AB688402	AB688562	AB688289
P. palmivora	4	C84[c]	Okinawa	2007	*Dendrobium phalaenopsis*	AB688403	AB688563	AB688290
P. porri	8b	MAFF 237664	Toyama	1997	*Allium victorialis*	AB688404	AB688564	AB688291
P. porri	8b	Porri-3	–	–	–	AB700471	AB700472	AB700473
P. porri	8b	MAFF 237666	Toyama	1997	Onion	AB688405	AB688565	AB688292
P. richardiae	8a	MAFF 235781	Chiba	1989	Calla lily	AB688406	AB688566	AB688293
P. richardiae	8a	CH 89-1	Chiba	1989	Calla lily	AB367379	AB688567	AB688294
P. richardiae	8a	CH 89PRK1	Kumamoto	1989	Calla lily	AB367380	AB688568	AB688295
P. sojae	7b	NBRC 31016	Shizuoka	1978	Soybean	AB217685	AB688569	AB688296
P. sojae	7b	Fuk12-2	Fukui	–	Soybean	AB688407	AB688570	AB688297
P. sojae	7b	Hok13-1	Hokkaido	–	Soybean	AB688408	AB688571	AB688298
P. syringae	8	MAFF 645010	Aomori	1991	*Malus pumila*	AB688409	AB688572	AB688299
P. tentaculata	1	C05	Chiba	2006	*Gazania* sp.	AB688410	AB688573	AB688300
P. vignae	7	Ph-9	Hokkaido	1977	Adzuki bean	AB367383	AB688574	AB688301
P. vignae	7	MAFF 241389	Hokkaido	2002	Adzuki bean	AB688425	AB688575	AB688302
Phytophthora sp.	1	MAFF 235784	Chiba	1989	*Vanda* sp.	AB688411	AB688576	AB688303
Phytophthora sp.	1	MAFF 235786	Chiba	1989	*Bougainvillea* sp.	AB688412	AB688577	AB688304
Phytophthora sp.	1	MAFF 235788	Chiba	1988	*Oncidium* sp.	AB688416	AB688581	AB688308
Phytophthora sp.	1	MAFF 235794	Chiba	1984	Chinese lantern	AB688414	AB688579	AB688306
Phytophthora sp.	2a	MAFF 238158	Kochi	1998	Ginger	AB688420	AB688585	AB688312
Phytophthora sp.	8b	MAFF 239556	Hyougo	2004	Lettuce	AB688417	AB688582	AB688309
Phytophthora sp.	1	GF468	Gifu	2003	Strawberry	AB688413	AB688578	AB688305
Phytophthora sp.	4	GF534	Gifu	2003	Fig	AB688415	AB688580	AB688307
Phytophthora sp.	2a	APC001	Aichi	2006	Winter melon	AB688421	AB688586	AB688313
Phytophthora sp.	7b	Toku-1	Toyama	2005	*Pueraria lobata*	AB688422	AB688587	AB688314

'–' Information is not available.

[a] Clade information.

[b] Abbreviations of isolates and culture collections with their sources: NBRC, NITE Biological Resource Centre, Japan; MAFF, Ministry of Agriculture, Forestry and Fisheries, Japan; CH, Chiba Prefectural Agriculture and Forestry Research Center, Chiba, Japan; GF, Gifu University, Gifu, Japan. Except, NBRC, MAFF.

[c] Okinawa Agricultural Research Centre isolates, all were our own isolates, identified based on morphological observations.

Table 2 – Primers used in this study for RNA amplification and sequencing.

Locus	Primer name	Primer sequence (5′–3′)	References
ITS + nLSU	UN_UP18S42	CGTAACAAGGTTTCCGTAGGTGAAC	Bakkeren et al. 2000
	UN_Lo28S576B	CTCCTTGGTCCGTGTTTCAAGACG	
ITS	ITS2[a]	GCTGCGTTCTTCATCGATGC	White et al. 1990
	ITS4[a]	TCCTCCGCTTATTGATATGC	
nLSU	NL1	GCATATCAATAAGCGGAGGAAAAG	O'Donnell 1993
	NL4	GGTCCGTGTTTCAAGACGG	
coxI	OomCoxI-Levlo	CYTCHGGRTGWCCRAAAAACCAAA	Robideau et al. 2011
	OomCoxI-Levup	TCAWCWMGATGGCTTTTTTCAAC	
TA cloning	M13M4	GTTTTCCCAGTCACGAC	
	M13Rv	CAGGAAACAGCTATGAC	

[a] Primers used for sequencing only.

or 2 μM of each primer (for *cox*I), 0.4 mg/ml BSA, 0.2 mM dNTPs (for ITS and nLSU) or 0.4 mM dNTPs (for *cox*I), 0.625 units of rTaq DNA polymerase (TaKaRa Bio, Otsu, Japan) and PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$). The PCR reactions were carried out in a 2700 DNA thermal cycler (Applied Biosystems). The ITS regions and nLSU were amplified together under the following conditions: 94 °C for 3 min followed by 35 cycles of 94 °C for 45 s, 68 °C for 45 s, and 72 °C for 90 s, with a final extension at 72 °C for 8 min. The nLSU was amplified alone using: 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The program for *cox*I was: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, with final extension at 72 °C for 10 min. All PCR products were checked for successful amplification by electrophoresis in 2% agarose gels (TAKARA L03 agarose, TaKaRa Bio).

2.2.3. *Sequencing reactions*

PCR products were purified using the GeneElute PCR clean-up kit (Sigma–Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. Each 10 μl sequencing reaction contained 1 μl purified PCR product, 1 μl primer (25-fold dilution of primer used in first PCR), 4 μl Ready Reaction Mix (Applied Biosystems) and 4 μl water. The thermocycler program was as follows: 96 °C for 1 min followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min. The sequencing reaction products were purified through ethanol precipitation then analyzed using an ABI 3100 DNA sequencer (Applied Biosystems). The sequences were edited using ChromasPro version 1.33 software (Technelysium Pty Ltd., Tewantin, Queensland, Australia), and the consensus sequences were used in alignments. The sequences were deposited in the DDBJ (http://ddbj.nig.ac.jp) GenBank database (Accession numbers shown in Table 1).

2.2.4. *TA cloning*

In some cases we did not obtain clear data by directly sequencing the PCR products. In such cases, the purified PCR products were inserted into the pT7Blue T vector (TaKaRa Bio) by TA cloning, according to the manufacturer's instructions. The cloned PCR products were sequenced as described above.

2.2.5. *Phylogenetic analysis and homology tests*

One reference isolate was selected for each species and taxon based on the results from the BLAST search at the NCBI. Ex-type and type cultures were given priority in selecting the isolates. In some cases, authentic isolates with more than 98% homology with the ex-type and type cultures were selected. We tried to include all species and taxa that have been described or are in the process of being described (Table S1). Homology tests were performed using GENETYX-WIN (Version 4.0) of the ITS sequences and the isolates were classified as having high or low homology with the ITS sequences of the reference isolates. In the first step of the phylogenetic analysis, a species-wise comparison was performed using the high homology isolates (data not shown). Second, a region-wise (ITS, nLSU, and *cox*I) phylogenetic analysis was performed using all 37 low homology isolates, to trace the positions of these isolates. This way, three phylogenetic trees were constructed (data not shown) for the three regions, as inferred by maximum parsimony (MP) and neighbor joining (NJ) with the PAUP version 4.0β10 software (Swofford 2002). In the third step, a clade-wise phylogenic tree was constructed using only the ITS sequence data, to clarify the exact position of each isolate (data not shown). Finally, a combined phylogenetic tree was constructed using the sequences of the ITS regions, nLSU and the *cox*I gene, inferred by NJ and MP (Fig. 1). In all the cases sequences was first aligned using the Clustal X multiple sequence alignment software (Thompson et al. 1997). The alignment has been deposited in TreeBASE as S13726.

2.3. *Evaluation of sequence variation*

The high homology isolates grouped together with their originally defined species in the ITS phylogenetic tree. However, we did find some intra-specific variations. For 19 of the 21 species included in the study, we analyzed the ITS regions, nLSU, and *cox*I gene sequences to identify the numbers of variable sites. We collected sequence data for the same three genetic regions in non-Japanese isolates of the 19 species, from the international *Phytophthora* Database (PD) (http://www.phytophthoradb.org/). The data were screened by making a phylogenetic tree using the ITS sequences, and sequences with low homology (<98%) with the ex-type and type cultures were discarded. The numbers of sequences in the PD were 369, 56, and 59 for the ITS regions, the nLSU, and the *cox*I gene, respectively. For reliable and dependable comparisons we only used isolates in which all three genetic regions had been sequenced, and thus our comparisons were limited by the numbers of nLSU and *cox*I gene sequences. We performed three alignments for each species: one including only Japanese isolates, one including only non-Japanese isolates, and the third including all isolates, and then counted the variable sites in each group (Table 5). We compared the data from two angles, one being variation in relation to geographic location (Japanese versus non-Japanese) and the other being variation within loci (ITS regions, nLSU, and *cox*I gene). Two of the 21 species, *P. megasperma* Drechsler and *P. chrysanthemi* Naher, Hid. Watanabe, Chikuo & Kageyama, were not included in the analysis because all Japanese isolates of *P. megasperma* were re-identified as different species, and there were no non-Japanese isolates of *P. chrysanthemi* in the PD. Locations or species with only a single accession were excluded from the comparative study.

3. Results and discussion

3.1. *Phylogenetic tree*

A phylogenetic analysis was performed using nuclear and mitochondrial DNA sequences from 161 Japanese *Phytophthora* isolates. These included 151 isolates representing 21 of the 31 species known to occur in Japan, and 10 unidentified isolates. The combined phylogenetic tree (Fig. 1) was similar to those produced in previous studies by Cooke et al. (2000) and Blair et al. (2008). The nLSU tree did not separate the isolates at the species level, and the following discussion is based on the

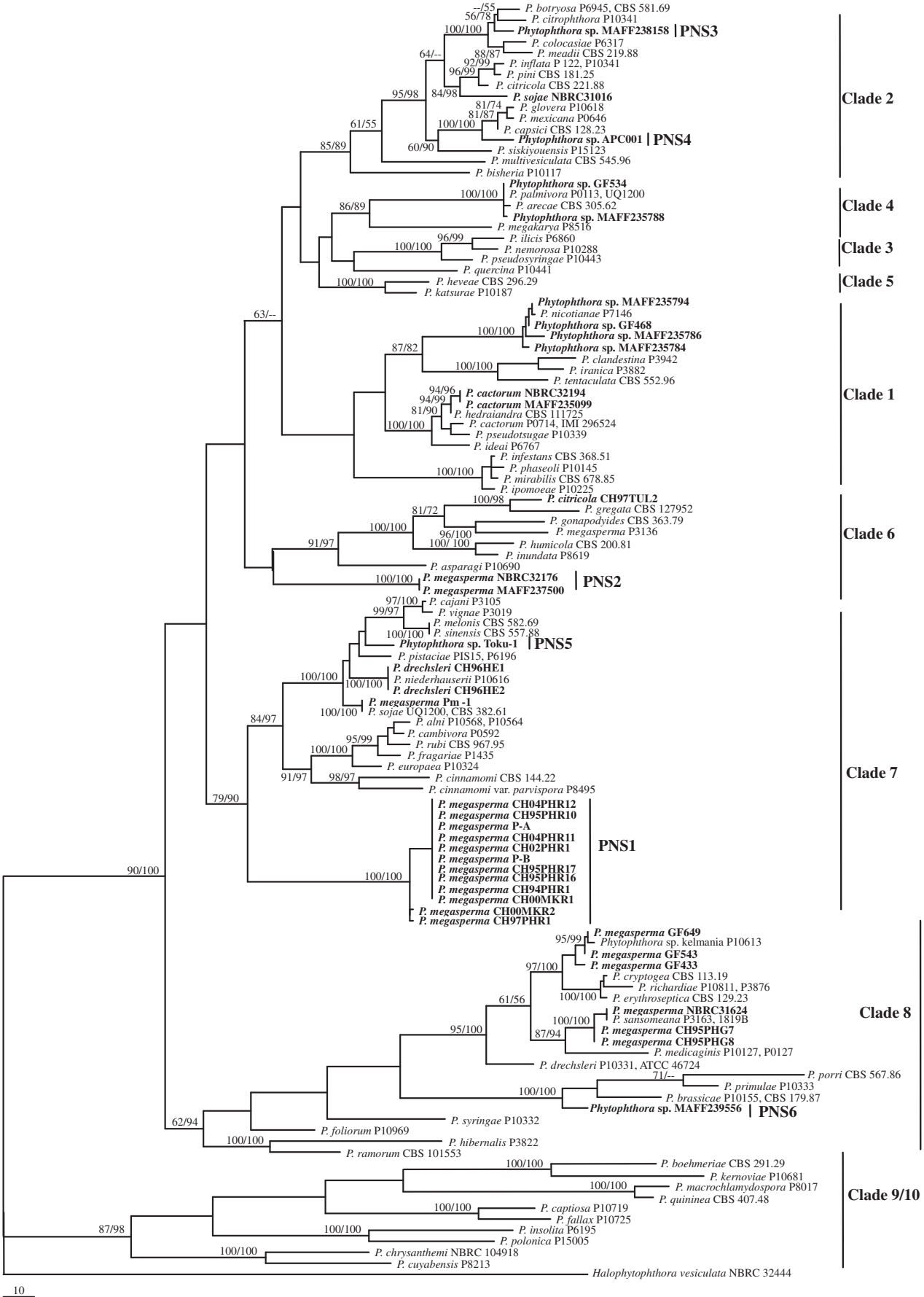

Table 3 – *Phytophthora* isolates that were unidentified or showed low homology with their originally identified species.

Isolate	Originally identified species	Homology %	Phylogenetically related species	Homology %
MAFF 235099	*P. cactorum* (IMI 296524)	99.2	*P. hedraiandra* (CBS 111725)	99.9
NBRC 32194	*P. cactorum* (IMI 296524)	99.2	*P. hedraiandra* (CBS 111725)	99.9
CH 97TUL2	*P. citricola* (CBS 221.88)	87.8	*P. gregata* (CBS 127952)	99.9
NBRC 31016	*P. sojae* (UQ1200)	89.6	*P. multivora* (CBS 124095)	99.6
CH 96HE1	*P. drechsleri* (ATCC 46724)	84.4	*P. niederhauserii* (P10616)	100
CH 96HE2	*P. drechsleri* (ATCC 46724)	84.4	*P. niederhauserii* (P10616)	100
CH 95PHG7	*P. megasperma* (P3136)	84.4	*P. sansomeana* (1819B)	99.1
CH 95PHG8	*P. megasperma* (P3136)	85.3	*P. sansomeana* (1819B)	99.2
NBRC 31624	*P. megasperma* (P3136)	85.4	*P. sansomeana* (1819B)	100
Pm-1	*P. megasperma* (P3136)	84.6	*P. sojae* (UQ1200)	99.9
GF433	*P. megasperma* (P3136)	85.8	*P.* sp. kelmania (P10613)	99.5
GF543	*P. megasperma* (P3136)	84.8	*P.* sp. kelmania (P10613)	99.7
GF649	*P. megasperma* (P3136)	85	*P.* sp. kelmania (P10613)	99.7
MAFF 235784	*Phytophthora* sp.		*P. nicotianae* (P7146)	99.4
MAFF 235786	*Phytophthora* sp.		*P. nicotianae* (P7146)	99.4
GF468	*Phytophthora* sp.		*P. nicotianae* (P7146)	99.5
MAFF 235794	*Phytophthora* sp.		*P. nicotianae* (P7146)	99.8
GF534	*Phytophthora* sp.		*P. palmivora* (UQ1294)	99.6
MAFF 235788	*Phytophthora* sp.		*P. palmivora* (UQ1294)	99.7

combined tree, which was constructed using the sequences of all three regions (ITS, nLSU and *cox*I) (Fig. 1).

Molecular phylogenetic analyses of *Phytophthora* were first performed by Cooke et al. (2000) who used the ITS regions. Later similar analysis was performed by Kroon et al. (2004) and Blair et al. (2008), using four and seven coding regions, respectively. Blair et al. (2008) first confirmed and consolidated the 10 clades in *Phytophthora*. These categories are now widely accepted (Kroon et al. 2012). The phylogenetic tree produced in this study was similar to the one produced by Blair et al. (2008), except that clades 9 and 10 were not clearly separated.

Among the 161 Japanese isolates analyzed in this study we did not find any that grouped with the species in clades 3, 5, and 10. Clade 3 consists of four species: *P. ilicis* Buddenhagen & Roy A. Young, *P. nemorosa* E. M. Hansen & Reeser, *P. pseudosyringae* T. Jung & Delatour, and *P. psychrophila* T. Jung & E. M. Hansen. Previously, *P. ilicis* was not common (Erwin and Ribeiro 1996), but recently it is reported to cause serious problem of holly in Spain (Pintos et al. 2012) and United Kingdom, and the other three species are newly described. Clade 5 consists of the species *P. heveae* A.W. Thompson, *P. katsurae* W. H. Ko & H. S. Chang and *P.* taxon agathis (Beever et al. 2009), and *P. katsurae* was previously reported in Japan by Katsura (1976). Clade 10 consists of *P. boehmeriae* Sawada, *P. kernoviae* Brasier, Beales & S. A. Kirk, *P. gallica* T. Jung & Nechwatal, and *P. morindae* Abad & S. C. Nelson. *Phytophthora boehmeriae* has been found on egg plants in Japan. The other three species in clade 10 are not widely distributed, and have been found only as tree pathogens: *P. kernoviae* in the UK and New Zealand (Érsek and Ribeiro 2010), *P. gallica* in France and Germany (Jung and Nechwatal 2008), and *P. morindae* in Hawaii (Nelson and Abad 2010). Despite the lack of isolates from these clades, our results suggest that phylogenetically diverse *Phytophthora* species can be found in Japan.

3.2. *Homology test*

Among the 161 isolates examined in this study, the ITS sequences of 124 isolates showed high homology with the ITS sequences from internationally representative isolates of known species. Twenty-seven isolates that had been identified at the species level showed low homology in their ITS regions with the representative isolates of those species. Of these 27 low homology isolates, 13 showed high homology (>99%) with species other than their originally identified species, and 14 showed low homology with all representative isolates of known species. In addition, we examined 10 isolates that had not been identified at the species level, and among these, 6 showed high homology with certain known species while 4 showed low homology with all representative isolates of known species. All these 37 isolates has shown in bold in Fig. 1

3.2.1. *Isolates showing high homology with species other than their originally identified species*

In this study, 13 isolates were grouped with species other than their originally identified species, and 6 previously unidentified isolates grouped with known species (Table 3). Except for one isolate that had been identified as *P. megasperma*, the newly defined species candidates were grouped into recently described species, which probably explains why they were originally misidentified.

Out of 15 isolates originally identified as '*P. cactorum* (Lebert & Cohn) J. Schröter', two isolates, NBRC 32194 and MAFF

Fig. 1 – Phylogenetic tree showing relationships among *Phytophthora* isolates based on the ITS regions, nLSU, and *cox*I gene sequences, inferred by maximum parsimony (MP). Node support values for MP (first) and neighbor joining (second) are shown side by side. Scale bar units: number of nucleotide substitutions per site. Clade categories correspond with those of Blair et al. (2008). The isolates shown in bold are the species names that were previously assigned on the basis of morphological criteria.

Table 4 – New species candidates of *Phytophthora*.

Originally identified species	Isolate number	Designated group[a]	Blair's clade[b]
P. megasperma	CH 04PHR11	PNS 1	Clade 7
P. megasperma	CH 04PHR12	PNS 1	Clade 7
P. megasperma	CH 94PHR1	PNS 1	Clade 7
P. megasperma	CH 95PHR10	PNS 1	Clade 7
P. megasperma	CH 95PHR16	PNS 1	Clade 7
P. megasperma	CH 95PHR17	PNS 1	Clade 7
P. megasperma	CH 00MKR1	PNS 1	Clade 7
P. megasperma	CH 00MKR2	PNS 1	Clade 7
P. megasperma	CH 02PHR1	PNS 1	Clade 7
P. megasperma	CH 97PHR1	PNS 1	Clade 7
P. megasperma	P-A	PNS 1	Clade 7
P. megasperma	P-B	PNS 1	Clade 7
P. megasperma	MAFF 237500	PNS 2	Clade 6
P. megasperma	NBRC 32176	PNS 2	Clade 6
Phytophthora sp.	MAFF 238158	PNS 3	Clade 2
Phytophthora sp.	APC001	PNS 4	Clade 2
Phytophthora sp.	Toku-1	PNS 5	Clade 7
Phytophthora sp.	MAFF 239556	PNS 6	Clade 8

[a] Designated group means, our assigned probable new species (PNS) groups.
[b] Phylogenetic tree constructed by Blair et al. (2008).

235099, from tulips in Niigata showed high homology with the ex-type culture of *P. hedraiandra* de Cock & Man in't Veld (CBS 111725), having one bp difference in the ITS regions. They showed closer relationships with *P. hedraiandra*, with support values of 94% in MP and 99% in the NJ tree (Fig. 1). The type culture of *P. cactorum* (IMI 296524) was positioned near *P. pseudotsugae* Hamm & E. M. Hansen. The two Japanese isolates of '*P. cactorum*' showed higher homology (99.9%) with *P. hedraiandra* (CBS 111725) than with *P. cactorum* (99.2%) in their ITS regions. However, the ITS regions of *P. hedraiandra* and *P. cactorum* are themselves quite homologous (99.4%). Although *P. hedraiandra* is closely related to *P. cactorum*, it was identified as a new species by de Cock and Lévesque (2004) based on the presence of tangled hyphae below the antheridia and large-sized oogonia. Up to now it has been reported as a pathogen of *Viburnum* spp. in the Netherlands, Spain and Italy, and *Rhododendron* sp. in Slovenia and USA (Érsek and Ribeiro 2010).

Among three isolates originally identified as '*P. citricola*', CH 97TUL2 grouped with *P. gregata* with high bootstrap support of 100% (Fig. 1). We found 99.9% homology with *P. gregata* (CBS 127952) and 87.8% with *P. citricola* (CBS 221.88). *Phytophthora gregata* is located in clade 6 of the phylogenetic tree and was isolated from forest soils in Australia (Burgess et al. 2009; Jung et al. 2011). A collection of this species has not been reported since its original description.

The '*P. sojae*' isolate NBRC 31016, from soybean in Shizuoka, showed high homology (99.6%) with *P. multivora* P. M. Scott & T. Jung (CBS 124095) and low homology (89.6%) with *P. sojae* (UQ1200). The nLSU sequence of *P. multivora* was not available in the PD, so it was not possible to include *P. multivora* in the combined phylogenic tree analysis. *Phytophthora sojae* NBRC 31016 grouped with *P. multivora* in the coxI and ITS trees (data not shown) with high bootstrap support (100% in NJ and 70.6% in MP). In the combined tree *P. sojae* NBRC 31016 grouped separately, basal to *P. citricola*. The results suggested that the isolate NBRC 31016 will be *P. multivora*. *Phytophthora multivora* was originally recovered from soil in declining and dead trees (Scott et al. 2009) and has not been reported since its original description. The NBRC 31016 isolate was reported to be pathogenic to soybean (Suzui 1983). There have been no reports on the pathogenicity of *Phytophthora* species in Japan, apart from *P. sojae*. Therefore, further studies will be necessary to classify this isolate as a new pathogen.

Out of five isolates identified as '*P. drechsleri* Tucker', CH 96HE1 and CH 96HE2, from ivy (*Hedera helix*) in Chiba, showed high homology (100%) with *P. niederhauserii* Z. G. Abad & J. A. Abad (P10616) and low homology (84.4%) with *P. drechsleri* (ATCC 46724). These two isolates were phylogenetically identified as *P. niederhauserii* with strong bootstrap support (100%) in the combined tree (Fig. 1). *Phytophthora niederhauserii* is a recently described species by Abad et al. (in press). The species has a wide distribution and host range.

Out of 21 isolates identified as '*P. megasperma*', three isolates, CH 95PHG7 and CH 95PHG8 from gerbera in Chiba, and NBRC 31624 from soil in Hokkaido, grouped with the newly described species *P. sansomeana* E. M. Hansen & Reeser with strong support of 100% (Fig. 1). The NBRC 31624 ITS sequence was 100% homologous with that of the authentic culture of *P. sansomeana* (1819B). Isolates CH 95PHG7 and CH 95PHG8 showed >99% homology with *P. sansomeana* and low homology (84–85%) with *P. megasperma* (P3136). *Phytophthora sansomeana* was segregated from *P. megasperma* by Hansen et al. (2009). It is a pathogen of soybean and Douglas-fir in the USA and of soybean in China (Érsek and Ribeiro 2010).

One isolate that was originally identified as '*P. megasperma*', Pm-1, from soybean in Hokkaido, showed high homology with *P. sojae*. Pm-1 grouped with *P. sojae* in clade 7 of the combined phylogenetic tree, with a high bootstrap value of 100% (Fig. 1). The isolate showed 99.9% homology with *P. sojae* (UQ1200) and low homology (84.6%) with *P. megasperma* (P3136). *Phytophthora sojae* was previously treated as *P. megasperma* var. *sojae*. However, Kaufmann and Gerdemann (1958) re-described the subspecies as *P. sojae*. After an argument of nomenclature, Faris et al. (1989) supported the name *P. sojae* and Hansen and Maxwell (1991) also accepted it.

Three isolates identified as '*P. megasperma*', GF433, GF543, and GF649, from gerbera in Gifu showed high homology with *P.* sp. kelmania (Abad et al. 2008). These isolates grouped with *P.* sp. kelmania in the combined phylogenic tree with bootstrap support of 95% in MP and 99% in NJ (Fig. 1). All of these isolates showed high homology (>99%) with *P.* sp. kelmania (P10613) and low homology (84–86%) with *P. megasperma* (P3136). This species was described by Abad et al. (2008), but is not a formal species.

Among the ten unidentified *Phytophthora* sp. isolates we were able to indicate four isolates, MAFF 235784, MAFF 235786, GF468, and MAFF 235794, from *Venda* sp., *Bougainvillea* sp., Chinese lantern, and strawberry, as *P. nicotianae* Breda de Haan using the phylogenetic analysis and the sequence homology search. *Phytophthora nicotianae* was clearly positioned within clade 1 along with *P. clandestina* P. A. Taylor, Pascoe & F. C. Greenhalgh, *P. iranica* Ershad, and *P. tentaculata* Kröber & Marwitz. This was consistent with the results of Cooke et al. (2000) but inconsistent with those of Blair et al. (2008), who

Table 5 – Variation among *Phytophthora* species in total numbers of base pairs and numbers of variable sites in the ITS regions, nLSU, and coxI gene sequences.

Species	Origin	ITS			nLSU			coxI		
		No. of isolates	Total length (bp)	No. of variable sites (bp)	No. of isolates	Total length (bp)	No. of variable sites (bp)	No. of isolates	Total length (bp)	No. of variable sites (bp)
P. cactorum	Japanese	13	792	5	13	710–711	3	13	672	8
	Other[a]	45	786–792	22	3	711	0	3	673	0
	Combined[b]	58	786–792	25	16	710–711	3	16	672–673	8
P. cambivora	Japanese	1	832	0	1	712	0	1	672	0
	Other[a]	16	829–833	25	3	712	3	2	674	0
	Combined[b]	17	829–833	25	4	712	3	3	672–674	0
P. capsici	Japanese	11	751–752	5	11	710–711	1	11	673	7
	Other[a]	52	750–753	54	7	711	0	4	672	4
	Combined[b]	63	750–753	58	18	710–711	1	15	672–673	8
P. cinnamomi	Japanese	7	826–830	2	7	711	0	7	672	0
	Other[a]	33	824–830	12	4	711	0	6	671–672	3
	Combined[b]	39	824–830	14	11	711	0	13	671–672	3
P. citricola	Japanese	2	761–820	6	2	710–711	0	2	672	12
	Other[a]	19	747–763	23	4	711	1	1	672	0
	Combined[b]	21	747–820	27	6	710–711	1	3	672	14
P. citrophthora	Japanese	7	780–783	7	7	710–711	0	7	672	7
	Other[a]	9	778–784	17	2	711	0	2	672	0
	Combined[b]	16	778–784	20	9	710–711	1	9	672	7
P. colocasiae	Japanese	1	779	0	1	710	0	1	672	0
	Other[a]	20	780–782	12	2	710	0	2	672	0
	Combined[b]	21	779–782	13	3	710	0	3	672	0
P. cryptogea	Japanese	15	797–803	19	15	709–712	8	15	672	21
	Other[a]	26	796–798	12	4	711	8	11	672	22
	Combined[b]	41	797–803	27	19	712	8	26	672	27
P. drechsleri	Japanese	3	798–826	1	3	708–710	2	3	672	2
	Other[a]	6	797–804	2	3	711	26	3	672	2
	Combined[b]	9	797–826	2	6	708–711	28	6	672	3
P. infestans	Japanese	2	795	0	2	710	0	2	672	0
	Other[a]	29	794–796	6	3	710	0	2	672	2
	Combined[b]	32	794–796	6	5	710	0	4	672	2
P. melonis	Japanese	3	820	1	3	710–711	0	3	672	0
	Other[a]	6	825–826	4	2	711	0	2	672	0
	Combined[b]	9	820–826	5	5	710–711	0	5	672	0
P. nicotianae	Japanese	32	802–807	24	32	709–710	1	32	670–674	7
	Other[a]	42	801–804	28	6	710	1	4	672	3
	Combined[b]	74	801–807	48	38	709–710	1	36	670–674	8
P. palmivora	Japanese	15	786–787	6	15	709–710	2	15	672–673	6
	Other[a]	27	783–790	18	2	710	0	4	672	44
	Combined[b]	42	783–790	19	17	709–710	2	19	672–673	51
P. porri	Japanese	3	805–806	3	3	711	0	3	672–673	1
	Other[a]	6	805	9	0	0	0	2	672	0
	Combined[b]	9	805–806	9	0	0	0	5	672–673	1

(*continued on next page*)

Table 5 – (continued)

Species	Origin	ITS			nLSU			coxI		
		No. of isolates	Total length (bp)	No. of variable sites (bp)	No. of isolates	Total length (bp)	No. of variable sites (bp)	No. of isolates	Total length (bp)	No. of variable sites (bp)
P. richardiae	Japanese	3	797–799	0	3	688	0	3	672	0
	Other[a]	6	796–797	2	5	712	0	1	672	0
	Combined[b]	9	796–799	2	8	688–712	3	4	672	0
P. sojae	Japanese	2	826	3	2	710–711	1	2	671–672	0
	Other[a]	8	825–826	3	1	711	0	4	672	4
	Combined[b]	10	825–826	5	3	710–711	1	6	671–672	4
P. syringae	Japanese	1	813	1	1	710–711	0	1	673	0
	Other[a]	14	812–813	1	2	710	1	2	672	2
	Combined[b]	15	812–813	2	3	710–711	1	3	672–673	3
P. tentaculata	Japanese	1	792	0	1	710	0	1	672	0
	Other[a]	3	791–792	1	2	710	0	2	672	0
	Combined[b]	4	791–793	1	3	710	0	3	672	0
P. vignae	Japanese	2	826	0	2	710–711	0	2	672	0
	Other[a]	2	814–826	0	1	711	0	2	672	0
	Combined[b]	4	814–826	0	3	710–711	0	4	672	0

[a] Sequence data from non-Japanese isolates.
[b] Combined data for Japanese and non-Japanese isolates.

found that the position of *P. nicotianae* was not clear but basal to clade 1. However, our four isolates grouped with *P. nicotianae* in clade 1 with high bootstrap support (100%), and showed high homology (>99%) with *P. nicotianae* (P7146). In addition, we found two previously 'unidentified isolates', GF534 and MAFF 235788, from *Oncidium* sp. and fig, respectively, grouped with *P. palmivora* (E. J. Butler) E. J. Butler along with *P. arecae* (L. C. Coleman) Pethybridge, with strong support (100%) (Fig. 1). These isolates also showed high homology (>99%) with the representative *P. palmivora* isolate UQ1294, which was used by Cooke et al. (2000). Both species are heterothallic in nature and require two compatible mating types to produce sexual structures, and are thus difficult to identify based on morphological structures.

3.2.2. *Isolates showing low homology with representative isolates of known species*

A total of 18 isolates, 14 that had been identified as *P. megasperma* and 4 that had previously been unidentified, showed low homology with all the internationally representative isolates of known species (Table 4).

Twelve of the 14 isolates, (CH 04PHR11, CH 04PHR12, CH 94PHR1, CH 95PHR10, CH 95PHR16, CH 95PHR17, CH 00MKR1, CH 00MKR2, CH 02PHR1, CH 97PHR1, P-A, and P-B), from roses in Chiba, Kanagawa, and Shizuoka, formed a monophyletic group in clade 7 with high bootstrap support (100%). This group was designated as "Probable New Species (PNS) 1". In their ITS regions, all of these 12 isolates showed low homology (82–84%) with that of '*P. megasperma*', which was their originally identified species (Fig. 1). Hansen et al. (1986) also suggested that the rose isolate P-A (Nagai et al. 1978) was different from *P. megasperma sensu strict* based on an isozyme analysis. Two of the 14 isolates, MAFF 237500 and NBRC 32176, which were isolated from easter lily in Kagoshima, were located in clade 6 of the phylogenetic tree and generated a monophyletic sub-clade. These are candidates for a new species, was designing as PNS2.

Hansen et al. (1986) recognized *P. megasperma* as a complex of distinct emerging biological species groups based on growth behavior, protein pattern, pathogenicity, karyotype and morphology (Hansen et al. 2009). Hansen and Maxwell (1991) segregated *P. sojae*, *P. medicaginis* E. M. Hansen & D. P. Maxwell, and *P. trifolii* E. M. Hansen & D. P. Maxwell, which are related to legume pathogens, from *P. megasperma sensu latu*. Furthermore, two new species, *P. rosacearum* E. M. Hansen & W. F. Wilcox and *P. sansomeana*, were recently established by Hansen et al. (2009). In this study, two groups of isolates that had previously been identified as *P. megasperma* appear to represent new species. On the other hand, *P. megasperma sensu strict* was not found among the Japanese isolates.

Four unidentified isolates from ginger, lettuce, winter melon and kudzu were located in clades 2, 8, 2, and 7 respectively, and generated monophyletic sub-clades, suggesting that they might be new species. In the phylogenic tree shown in Fig. 1, the unidentified *Phytophthora* sp. MAFF 238158 was positioned in clade 2 with *P. citrophthora* and *P. botryosa* Chee with low bootstrap support. In clade 2 of the separate ITS tree, this isolate showed close relationships with *P. colocasiae* Raciborski and *P. meadii* McRae (data not shown). The position of MAFF 238158 was ambiguous, might be a candidate for a new species that we designated as PNS3.

The position of *Phytophthora* sp. APC001 was also not clear in the phylogenetic analysis. Although APC001 was positioned separately in the combined MP tree (Fig. 1), it showed a close relationship with *P. capsici* Leonian in clade 2 of the separate ITS tree (data not shown). Therefore, APC001 might be a new species designated as PNS4 in this study.

Phytophthora sp. Toku-1 was positioned separately in clade 7 (Fig. 1). In its ITS sequence, this isolate showed 99.5%, 99.4%, and 98.5% homology with those of *P. sojae* (UQ1200), *P. niederhauserii* (P10616) and *P. pistaciae* Mirabolfathy (PIS15), respectively. The Toku-1 isolate might be a new species that we designated as PNS5.

Phytophthora sp. MAFF 239556 was positioned in clade 8 with the closely related species *P. brassicae* de Cock & Man in't Veld (Fig. 1). We found 98.6% homology with *P. brassicae* (CBS 179.87) in the ITS regions. The MAFF 239556 isolate might be a new species, designated as PNS6 in this study.

3.3. *Intra-specific variations*

In the isolates we studied, the ITS regions and the *cox*I genes were more variable than the nLSU coding regions, and there was no relationship between variability and geographic location (i.e., Japanese versus non-Japanese) (Table 5). In the ITS sequences, relatively high numbers of variable sites were observed in *P. capsici* (58), *P. nicotianae* (48), *P. citricola* (27), *P. cryptogea* Pethybridge & Lafferty (27), *P. cactorum* (25), *P. cambivora*(Petri) Buisman (25), *P. citrophthora* (20), *P. palmivora* (19), *P. cinnamomi* Rands (14), and *P. colocasiae* (13). No variable sites were present in *P. vignae*, irrespective of location. In the *cox*I gene sequences, relatively high numbers of variable sites were found in *P. palmivora* (51), *P. cryptogea* (27), *P. citricola* (14), *P. cactorum* (8), *P. capsici* (8), *P. nicotianae* (8) and *P. citrophthora* (7). In contrast, *P. cambivora*, *P. colocasiae*, *P. melonis* Katsura, *P. richardiae* Buisman, *P. tentaculata* and *P. vignae* Purss did not show any variable sites, irrespective of location. *Phytophthora drechsleri* showed highest number of variable sites (28) in the nLSU sequence, and was followed by *P. cryptogea*, with 8 variable sites. Considering all locations and loci *P. cryptogea* *P. nicotianae*, *P. palmivora*, *P. capsici*, *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citricola*, and *P. citrophthora* showed comparatively high variability. Apart from *P. citricola,* all these species were heterothallic.

There were no significant differences between the Japanese and non-Japanese isolates in terms of the numbers of variable sites. In some cases there was more variation within either the Japanese or the non-Japanese isolates of a particular species at all three loci, however, this might be due to variations in the numbers of isolates studied. Uddin et al. (2007) studied intra-isolate variations within *P. cryptogea* and found similar results.

Along with the ITS sequences, the *cox*I gene sequences were more variable than the nLSU sequences in *P. cryptogea*, *P. nicotianae*, *P. palmivora*, *P. capsici*, *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citrophthora*, and *P. citricola*. In some cases, the *cox*I sequences showed equal (*P. cryptogea*) or higher (*P. palmivora*) variability than the ITS sequences. In most species, number of variable sites of ITS regions is more than those of *cox*I genes. Regardless, the variability in the ITS region and in the *cox*I gene is very important for species identification, especially for the heterothallic species.

The use of DNA sequences for oomycete species identification is well established, and the use of *cox*I as a DNA barcoding is a new approach (Robideau et al. 2011). In our analysis, we found that species identification using *cox*I is a practical option. This approach complements the use of the ITS sequences, because it uses the mitochondrial genome instead of nuclear RNA. In some cases, the *cox*I gene was more discriminative than the ITS and nLSU regions at the species level. For example, our nLSU tree did not separate the *P. drechsleri* isolates CH 96HE1 and CH 96HE2 from *Phytophthora* sp. Toku-1, *P. niederhauserii*, and *P. sojae*; however, the *cox*I tree did. This was also the case for the *P. cactorum* isolates NBRC 32194 and MAFF 235099. Similar results were found by Robideau et al. (2011), who proposed that both the *cox*I and ITS sequences are acceptable and complementary DNA barcodes for the identification of Oomycetes. We also support this idea.

This re-evaluation of the *Phytophthora* species in Japanese culture collections has clarified the identities of the previously morphologically assigned isolates and identified six possible new species. We have also provided information about how some misidentifications may have occurred, and offered possible remedies. In addition, our variation analysis has contributed information about the diversity of Japanese *Phytophthora* species, which will be helpful in the general study of this genus, especially in Japan. Further detailed studies are necessary to confirm and clarify the positions of the probable new species candidates.

Disclosure

All co-authors are agreed with the content of the manuscript and there are no conflicts to report. We certify that all experiments complied with the current laws of Japan. This submission represents original work and is not under review in any other publication.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.myc.2013.11.005.

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