UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Administrative unit: **University of Padova**

Department: **Land, Environment, Agriculture and Forestry (TESAF)**

PhD Program: **Land, Environment, Resources and Health (LERH)**

Batch: XXVI

Ecological impact, epidemiology and genetic diversity of invasive *Phytophthora* species in mountain ecosystems

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Title

Ecological impact, epidemiology and genetic diversity of invasive *Phytophthora* species in mountain ecosystems

Abstract

The progressive introduction of alien and invasive pathogens in natural ecosystems represents one of the main threats to forest health worldwide. Among the main destructive plant pathogens accidentally introduced to new areas every year by the trade in plants and goods, deforestation and reforestation activity, change in land use, irrigation systems, urbanization and recreative forest activity, many belong to the genus *Phytophthora*. This oomycete genus currently encompasses over 220 species grouped into 12 well-defined phylogenetic clades. The global biodiversity of *Phytophthora* species has been extensively investigated over the last decades with the description of about a hundred new species. Notably, the development of molecular tools phylogenetic analysis has revealed an unexpected diversity of species in both natural and agricultural ecosystems in temperate and tropical areas. Despite the intense research activity on this group of pathogens worldwide, the presence and impact of *Phytophthora* species in mountain ecosystems remain underreported leaving a significant gap in our knowledge. Therefore, the main goal of this PhD project was to explore the diversity of *Phytophthora* species across different types of mountain and alpine forests in Europe in order to fill the gaps on the occurrence, distribution and ecological impact of these emerging pathogens on mountain vegetation. Field surveys conducted in different Italian, Portuguese and Slovenian forests over a three-year period showed a complex of 26 pathogenic *Phytophthora* species associated with leaf and shoot blights, bleeding cankers and root rot symptoms on 41 plant species typical of mountain vegetation. The variety of symptoms observed on plants were compatible with both air and soil-borne *Phytophthora* infections. The extensive field surveys conducted in this study highlight that severe disease outbreaks and mortality events are currently affecting shrubs and trees especially along riparian habitats. *Phytophthora pseudosyringae* and *P. plurivora* were the dominant species in the monitored sites, in particular, *P. pseudosyringae* was prevalent in the cold environments. Overall the species from clades 2 and 6 were the most common; they were recovered from different types of vegetation, streams and riparian ecosystems. *Phytophthora* communities found in the mountain vegetation show an unexpected highest diversity, including two new species namely *P. pseudogregata* and *P. heteromorpha*. A further new species, *P. mediterranea,* was discovered from myrtle plants grown in nurseries, this highlights the increasing risk posed by nursery material to

natural ecosystems. The riparian habitats were identified as the major natural corridors correlated with the diffusion of *Phytophthora* species in mountain ecosystems. In conclusion, this study contributes to expand knowledge on the ecology of *Phytophthora* species in both natural areas and nurseries with 87 new host-pathogens associations and 10 new *Phytophthora* reports for Italy, 11 for Portugal and 6 for Slovenia.

Introduction

Globally, mountain forests provide a wide range of services and resources to human societies such as fresh water, timber, climate regulation and recreation (Schirpke, 2022). Although mountains occupy only 12% of the planet's surface, the complex system of habitats and micro-habitats and the limited impact of human activities contribute to a quarter of global biodiversity and one-third of all protected areas (Körner & Paulsen, 2004, Chapes *et al*., 2008; Körner *et al*., 2011).

Among the major mountain ranges of the world, the European Alps represent one of the most important. The Alps are the highest and longest European mountain system. The entire range develops for about 1300 km, extending in width between 120 and 250 km and stretching over an area of approximately 300000 km^2 across eight countries. The Alps are characterized by a strong topographic variability, extensive lowlands and highlands and deep valleys and peaks; the average altitude is 1300 m a.s.l. but hundreds of peaks reach more than 3000/4000 m, progressively decreasing from west to east (Sergio & Pedrini, 2008; Nared *et al*., 2015).

The European mountain flora includes more than 13000 plant species, with many endemisms and a huge variety of habitats. The number of vascular plants in the Alps reaches about 40% of the total continental flora. Floral diversity in the Alps is generally linked to the geology, climate and vegetation conditions, and is distinguished by three main ideal bio-geographic regions: the Alpine fringe, dominated by *Fagus sylvatica*, the inter-Alpine zone by *Abies alba* and the continental poles progressively populated by *Picea abies*, *Larix decidua* and *Pinus cembra* (Ozenda, 1988). Above the tree line the vegetation is composed of a huge number of shrubs, heathland and herbal species, variable in composition based on the soil, slope orientation and climatic conditions (Körner, 2003).

In mountain ecosystems, biotic interactions and biodiversity decrease progressively with elevation (Alexander *et al*., 2018). It is possible to quantify the reduction in biodiversity as ca. 40 plant species each 100 m of altitude (Korner *et al*., 2011). Reductions in species richness are mainly caused by different climatic conditions, space and phenology limitations, such as adaptation to colder temperatures (Körner, 2004), the reduction in land area and space available to habitats and ecological niches (Pauchard *et al*., 2009), a limited functional space caused by restricted migration of different

species (Körner, 2003, Pauchard *et al*., 2009), the reduced vegetational season length caused by extreme temperatures, late and early frosts and the environmental physiological filtering due to progressive decline of species (Gerhardt & Collinge 2007, Alexander *et al*., 2011, Redondo *et al*., 2018). Along the altitudinal gradient, the tree line represents the fundamental point that divides large ground cover areas from a partially or totally uncovered one, progressively reducing the ecological niches. Beyond the tree line, trees cannot tolerate the environmental conditions such as cold temperatures, extreme snow cover, or associated lack of available moisture. The tree line often appears well-defined, but it can be profoundly influenced by human activities, such as high-altitude pasture (Körner, 2003).

Similarly to other natural environments, the stability and biodiversity of Alpine forests are threatened by a variety of adverse natural and human-mediated factors (Bengtsson *et al*., 2000; Senf & Seidl, 2021). Among the main disturbing factors, the accidental introduction of exotic fungal pathogens through the trade in plants and goods assumes a role of primary importance (Liebhold *et al.,* 2017; Wingfield *et al*., 2017). Over the past centuries the geographic barriers that for millennia helped to maintain the distribution of living organisms almost static within the continents have gradually been eroded by human activities (Richardson *et al*., 2000). In particular, global trade has facilitated the movement of plant material between countries and continents, increasing the exposure of native plants to new pathogens and pests (Brasier, 2008; Wingfield *et al.*, 2017). Furthermore, the lack of adequate prevention measures makes nursery plants for restoration the potential vehicle to introduce pathogens into natural environments (Brasier, 2008; Frankel *et al*., 2020; Antonelli *et al*., 2022). The constant introduction of invasive plant pathogens is threatening the economy of the forestry sector, the balance of ecosystems and survival of many plant species (Strange & Scott, 2005; Boyd *et al*., 2013).

The dispersion of fungal and like-fungal organisms in new areas has, in some cases, altered the bio-ecology of invasive organisms, such as host range, survival strategies and mechanisms of reproduction. Among the most destructive pathogens accidentally introduced in new areas every year by the trade in plant and goods, deforestation and reforestation activity, change in land use, irrigation systems, urbanization and recreative forest activity, many belong to the genus *Phytophthora* (Erwin & Ribeiro, 1996; Hansen, 2008; Yang *et al*., 2017; Scott *et al*., 2019a; Antonelli *et al*., 2022). This oomycete genus currently encompasses over 220 species grouped into 12 well-defined phylogenetic clades (Abad *et al*., 2023; Bregant *et al*., 2023). Since the description of *P. infestans* in 1876 the number of new described species has progressively increased. The largest number of new *Phytophthora* species in a single country have been described in the US, with 50 *taxa* found in natural,

agricultural and horticultural systems (Fig. 1 and Tab. 1). Regarding natural ecosystems, the higher number of new *Phytophthora* species was discovered in Australia and New Zealand, reaching over 30 species (Dick *et al*., 2006; Burgess *et al*., 2009, 2017a, 2018; Rea *et al*., 2010, 2011; Jung *et al*., 2011; Dunstan *et al*., 2016; Belhaj *et al*., 2018; Khaliq *et al*., 2018). In addition to Oceania, numerous studies have permitted 32 new *Phytophthora* species from European forests to be described. Considering all sectors (agriculture, horticulture and forestry), a total of 64 species have been described in Europe (Brasier & Jung, 2006; Jung & Burgess, 2009; Scanu *et al.*, 2015; Bregant *et al*., 2021a, 2021b).

Although the true diversity of *Phytophthora* is still unknown, in some continents the number of known species and new reports is quite large. Instead, in others, such as South America, Asia and especially Africa, new species description has been much lower than in Europe, Oceania and North America, stressing that further studies are needed to investigate the real diversity of these organisms (Fig. 1).

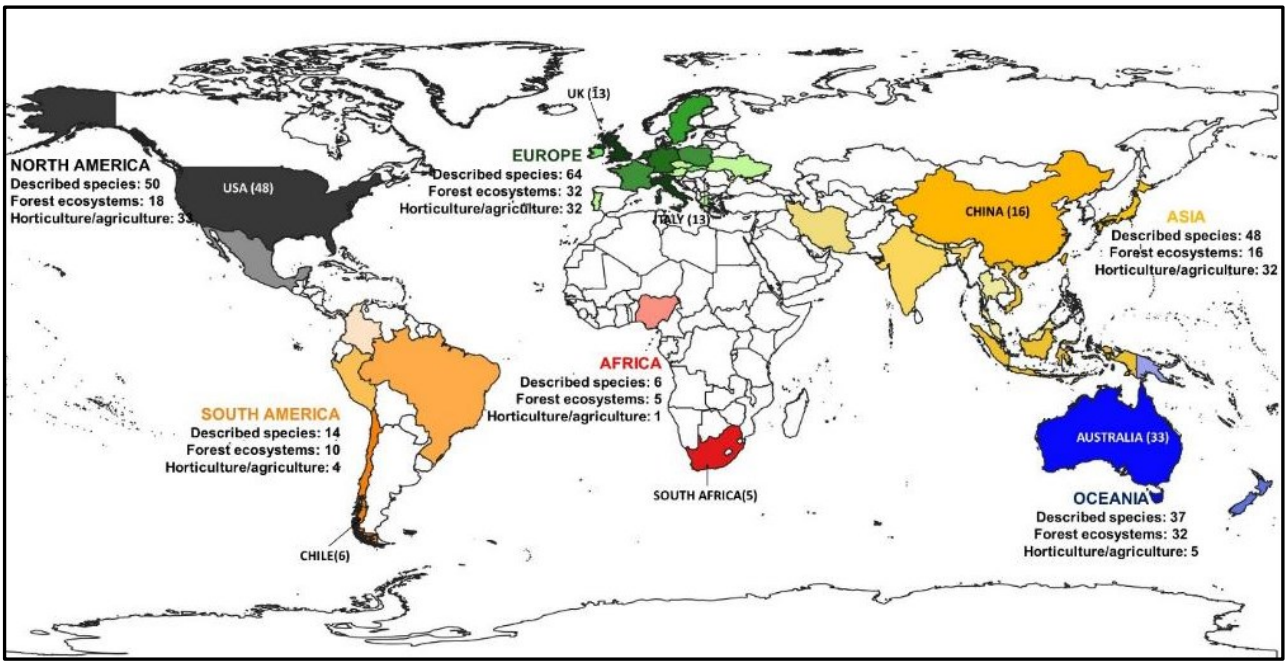

Figure 1. Number of *Phytophthora* species described per countries and continents.

Table 1. *Phytophthora* species described worldwide since 1998.

Year	Species	Clade	Breeding systems	Sporangia features	Host	Pathogenicity	Country	Reference
1998	*P. multivesiculata*	2	Homothallic	Persistent	*Cymbidium* sp.		Netherlands	Ilieva *et al.*, 1998
1999	*P. quercina*	12	Homothallic	Persistent	*Quercus robur*	Confirmed	Germany	Jung *et al.*, 1999
2001	*P. oryzo-bladis*	n/d	-	-	*Oryza sativa*	Confirmed	China	Ho, 2001

2001	*P. pistaciae*	7	Homothallic	Persistent	*Pistacia vera*	Confirmed	Iran	Mirabolfathy *et al*., 2001
2001	*P. ramorum*	8	Heterothallic	Caducous	*Rhododendron catawbiense*	Confirmed	Germany	Werres *et al.,* 2001
2001	*P. tropicalis*	2	Heterothallic	Caducous	*Macadamia integrifolia*	Confirmed	USA	Aragaki & Uchida, 2001
2002	*P. brassicae*	8	Homothallic	Persistent/ caducous	*Brassica oleracea*	Confirmed	Netherlands	Man in 't Veld *et al.,* 2002
2002	*P. europaea*	7	Homothallic	Persistent	*Q. robur*	Confirmed	France	Jung *et al*., 2002
2002	*P. ipomoeae*	1	Homothallic	Caducous	*Ipomoea longipedunculata*	Confirmed	Mexico	Flier et al., 2002
2002	*P. psychrophila*	3	Homothallic	Persistent/ caducous	*Q. robur*	Confirmed	Germany	Jung *et al*., 2002
2002	*P. uliginosa*	7	Homothallic	Persistent	*Q. robur*	Confirmed	Poland	Jung *et al*., 2002
2003	*P. inundata*	6	Sterile	Persistent	*Salix matsudana*	Confirmed	UK	Brasier *et al.,* 2003a
2003	*P. nemorosa*	3	Homothallic	Caducous	*Lithocarpus densiflorus*	Confirmed	USA	Douhan, & Rizzo, 2003
2003	*P. pseudosyringae*	3	Homothallic	Persistent/ caducous	*Q. robur*	Confirmed	Germany	Jung *et al*., 2003
2004	*P. hedraiandra*	1	Homothallic	Caducous	*Viburnum* sp.	Confirmed	Netherlands	De Cock *et al*., 2004
2005	*P. kernoviae*	10	Homothallic	Caducous	*Fagus sylvatica*	Confirmed	UK	Brasier *et al*., 2005
2006	*P. captiosa*	10	Homothallic	Persistent	*Eucalyptus* sp.		New Zealand	Dick *et al.,* 2006
2006	*P. fallax*	10	Homothallic	Persistent	*Eucalyptus delegatensis*		New Zealand	Dick *et al.,* 2006
2006	*P. foliorum*	8	Homothallic	Persistent	*Rhododendron* sp.	Confirmed	USA	Donahoo *et al*., 2006
2006	*P. polonica*	6	Homothallic	Persistent	*Alnus glutinosa*	Confirmed	Poland	Belbahri *et al*., 2006
2007	*P. alticola*	4	Homothallic	Persistent	*Eucalyptus grandis*	Confirmed	South Africa	Maseko *et al*., 2007
2007	*P. austrocedrae*	8	Homothallic	Persistent	*Austrocedrus chilensis*	Confirmed	Chile	Greslebin *et al*., 2007
2007	*P. frigida*	2	Heterothallic	Persistent	*Eucalyptus smithii*	Confirmed	South Africa	Maseko *et al*., 2007
2007	*P. litchii*	4	Homothallic	Caducous	*Litchi chinensis*		Taiwan	Göker *et al*., 2007
2007	*P. rubi*	7	Homothallic	Persistent	*Rubus idaeus*	Confirmed	UK	Man in 't Veld, 2007
2008	*P. asparagi*	6	Homothallic	Non papillate, persistent	*Asparagus officinalis*	Confirmed	USA	Saude *et al.,* 2008
2008	*P. bishii*	2	Homothallic	Persistent	*Fragraria ×ananassa*	Confirmed	USA	Abad *et al*., 2008
2008	*P. gallica*	10	Sterile	Persistent	*Quercus robur*	Confirmed	France	Jung & Nechwatal, 2008
2008	*P. irrigata*	9	Heterothallic	Non papillate, persistent	Water		USA	Hong *et al.,* 2008
2008	*P. parsiana*	9	Heterothallic	Persistent	*Ficus carica*		Iran	Mostowfizadeh-Ghalamfarsa *et al.,* 2008
2008	*P. pinifolia*	6	Sterile	Persistent	*Pinus radiata*	Confirmed	Chile	Durán *et al*., 2008
2008	*P. quercetorum*	4	Homothallic	persistent	*Quercus rubra*		USA	Balci *et al*., 2008
2008	*P. siskiyouensis*	2	Homothallic	Persistent/ caducous	*Lithocarpus densiflorus*	Confirmed	USA	Reeser *et al*., 2008
2009	*P. mengei*	2	Homothallic	Persistent	*Persea americana*	Confirmed	USA	Hong *et al*., 2009
2009	*P. multivora*	2	Homothallic	Persistent	*Eucalyptus marginata*	Confirmed	Australia	Scott *et al*., 2009
2009	*P. plurivora*	2	Homothallic	Persistent	*Q. robur*	Confirmed	Germany	Jung & Burgess, 2009
2009	*P. rosacearum*	6	Homothallic	Persistent	*Malus domestica*	Confirmed	USA	Hansen *et al.,* 2009
2009	*P. sansomeana*	8	Homothallic	Persistent	*Glycine max*	Confirmed	USA	Hansen *et al*., 2009
2010	*P. capensis*	2	Homothallic	Persistent	*Curtisia dentata*		South Africa	Bezuidenhout *et al.,* 2010

2010	*P. elongata*	2	Heterothallic	Persistent	*Eucalyptus marginata*	Confirmed	Australia	Rea *et al*., 2010
2010	*P. hydropathica*	9	Heterothallic	Persistent	*Rhododendron catawbiense*	Confirmed	USA	Hong *et al*., 2010
2010	*P. morindae*	10	Heterothallic	Caducous	*Morinda citrifolia*	Confirmed	USA	Nelson & Abad, 2010
2011	*P. arenaria*	4	Homothallic	Persistent	*Banksia attenuata*	Confirmed	Australia	Rea *et al*., 2011
2011	*P. chrysanthemi*	9	Homothallic	Persistent	*Chrysanthemum ×morifolium*	Confirmed	Japan	Naher *et al.,* 2011
2011	*P. constricta*	10	Homothallic	Persistent	*Banksia cirsioides*	Confirmed	Australia	Rea *et al*., 2011
2011	*P. fluvialis*	6	Sterile	Persistent	Water		Australia	Jung & Burgess, 2011
2011	*P. gemini*	6	Sterile	Persistent	*Zostera marina*		Netherlands	Man in ’t Veld *et al.* 2011
2011	*P. gibbosa*	6	Homothallic	Persistent	*Acacia pycnantha*		Australia	Jung *et al*., 2011
2011	*P. glovera*	2	Homothallic	Persistent	*Nicotiana tabacum*	Confirmed	Brazil	Abad *et al.*, 2011
2011	*P. gregata*	6	Homothallic	Persistent	*Patersonia* sp.		Australia	Jung *et al*., 2011
2011	*P. himalsilva*	2	Homothallic	Persistent/ caducous	*Quercus leuchotricophora*		Nepal	Vettraino *et al*., 2011
2011	*P. litoralis*	6	Sterile	Persistent	*Banksia* sp.		Australia	Jung *et al*., 2011
2011	*P. obscura*	8	Homothallic	Persistent	*Aesculus hippocastanum*	Confirmed	Germany	Grünwald *et al*., 2012
2011	*P. pini*	2	Homothallic	Persistent	*Pinus resinosa*	Confirmed	USA	Hong *et al.,* 2011
2011	*P. thermophila*	6	Homothallic	Persistent	*Eucalyptus marginata*		Australia	Jung *et al*., 2011
2012	*P. amnicola*	6	Homothallic	Persistent	Water	Confirmed	Australia	Crous *et al.,* 2012
2012	*P. aquimorbida*	9	Homothallic	Persistent	*Rhododendron* sp.	Confirmed	USA	Hong *et al.,* 2012
2012	*P. bilorbang*	6	Homothallic	Persistent	*Rubus anglocandicans*	Confirmed	Australia	Aghighi *et al*., 2012
2012	*P. borealis*	6	Sterile	Persistent	Water		USA	Hansen *et al.,* 2012
2012	*P. riparia*	6	Sterile	Persistent	Water		USA	Hansen *et al.,* 2012
2012	*P. ×pelgrandis*	1	Homothallic	Caducous	*Pelargonium grandiflorum*		Germany	Man in 't Veld *et al*., 2012
2012	*P. ×serendipita*	1	Homothallic	Caducous	*Idesia polycarpa*	Confirmed	Netherlands	Man in 't Veld *et al*., 2012
2013	*P. cichorii*	8	Heterothallic	Persistent	*Cichorium intybus*		Netherlands	Bertier *et al.*, 2013
2013	*P. dauci*	8	Homothallic	Persistent	*Daucus carota*		France	Bertier *et al.*, 2013
2013	*P. lactucae*	8	Homothallic	Persistent	*Lactuca sativa*		Greece	Bertier *et al.*, 2013
2013	*P. lacustris*	6	Sterile	Persistent	*Salix matsudana*	Confirmed	UK	Nechwatal *et al*., 2013
2013	*P. mississippiae*	6	Self-sterile	Persistent	Water		USA	Yang *et al.,* 2013
2013	*P. pisi*	7	Homothallic	Persistent	*Pisum sativus*	Confirmed	Sweden	Heyman *et al*., 2013
2013	*P. pluvialis*	3	Homothallic	Caducous/ persistent	Water	Confirmed	USA	Reeser *et al*., 2013
2014	*P. virginiana*	9	Sterile	Persistent	Water		USA	Yang & Hong, 2014
2014	*P. acerina*	2	Homothallic	Persistent	*Acer pseudoplatanus*	Confirmed	Italy	Ginetti *et al*., 2014
2014	*P. asiatica*	7	Homothallic	Persistent	*Pueraria lobata*	Confirmed	Japan	Rahman e*t al.*, 2014a
2014	*P. fragariaefolia*	7	Homothallic	Persistent	*Fragraria ×ananassa*	Confirmed	Japan	Rahman e*t al.*, 2014b
2014	*P. hydrogena*	9	Heterothallic	Persistent	Water		USA	Yang *et al.,* 2014a
2014	*P. macilentosa*	9	n/d	Persistent	Water		USA	Yang *et al.,* 2014b
2014	*P. moyootj*	6	Sterile	Persistent	Mud		Australia	Crous *et al*., 2014

2014	*P. nagaii*	7	Homothallic	Persistent	*Rosa* sp.	Confirmed	Japan	Rahman e*t al.*, 2014b
2014	*P. niederhauserii*	7	Heterothallic	Persistent	*Hedera helix*	Confirmed	USA	Abad *et al.*, 2014
2014	*P. pachypleura*	2	Homothallic	Persistent	*Aucuba japonica*	Confirmed	UK	Henricot *et al.,* 2014
2014	*P. parvispora*	7	Heterothallic	Persistent	*Arbutus unedo*	Confirmed	Italy	Scanu *et al*., 2014a
2014	*P. stricta*	n/d	Heterothallic	Caducous	Water		USA	Yang *et al.,* 2014b
2014	*P. ×stagnum*	6	Heterothallic	Persistent	Water		USA	Yang *et al*., 2014c
2015	*P. agathidicida*	5	Homothallic	Persistent	*Agathis australis*	Confirmed	New Zealand	Weir *et al*., 2015
2015	*P. amaranthi*	2	Homothallic	persistent	*Amaranthus tricolor*	Confirmed	Taiwan	Ann *et al*., 2016
2015	*P. boodjera*	4	Homothallic	Persistent	*Eucalyptus grandis*	Confirmed	Australia	Simamora *et al*., 2015
2015	*P. chlamydospora*	6	Sterile	Persistent	*Prunus* sp.	Confirmed	UK	Hansen *et al*., 2015
2015	*P. cocois*	5	Homothallic	Persistent	*Cocos nucifera*		USA	Weir *et al*., 2015
2015	*P. crassamura*	6	Homothallic	Persistent	*Juniperus phoenicea*	Confirmed	Italy	Scanu *et al*., 2015
2015	*P. gondwanensis*	10	Homothallic	Caducous	Soil		Australia	Crous *et al*., 2015
2015	*P. lilii*	11	Homothallic	Persistent	*Lilium longiflorum*	Confirmed	Japan	Rahman et *al.,* 2015
2015	*P. occultans*	2	Homothallic	Caducous	*Buxus sempervirens*	Confirmed	Netherlands	Man In 't Veld *et al*., 2015
2015	*P. ornamentata*	6	Homothallic	Persistent	*Pistacia lentiscus*	Confirmed	Italy	Scanu *et al.,* 2015
2015	*P. pseudocryptogea*	8	Heterothallic	Persistent	*Isopogon buxifolius*	Confirmed	Australia	Safaiefarahani *et al*., 2015
2015	*P. terminalis*	2	Homothallic	Caducous	*Pachysandra terminalis*	Confirmed	Netherlands	Man In 't Veld *et al*., 2015
2015	*P. uniformis*	7	Homothallic	Persistent	*A. glutinosa*	Confirmed	Sweden	Husson *et al.,* 2015
2015	*P. ×alni*	7	Homothallic	Persistent	*A. glutinosa*	Confirmed	UK	Husson *et al.,* 2015
2015	*P. ×multiformis*	7	Homothallic	Caducous/ persistent	*A. glutinosa*	Confirmed	Netherlands	Husson *et al.,* 2015
2016	*P. caryae*	2	Homothallic	Persistent	Water		USA	Brazee *et al*., 2016
2016	*P. estuarina*	9	Sterile	Persistent	*Laguncularia racemosa*		Brazil	Li *et al*., 2016
2016	*P. intercalaris*	10	Homothallic	Persistent/ caducous	Water		USA	Yang *et al*., 2016
2016	*P. rhizophorae*	6	Sterile	Persistent	Water		Brazil	Li *et al*., 2016
2017	*P. attenuata*	7	Homothallic	Persistent	*Castanopsis carlesii*		Taiwan	Jung *et al*., 2017a
2017	*P. castanetorum*	12	Homothallic	Persistent	*Castanea sativa*	Confirmed	Portugal	Jung *et al*., 2017b
2017	*P. flexuosa*	7	Homothallic	Persistent	*Fagus hayatae*		Taiwan	Jung *et al*., 2017a
2017	*P. formosa*	7	Homothallic	Non papillate, persistent	*Araucaria cunninghamii*		Taiwan	Jung *et al*., 2017a
2017	*P. intricata*	7	Homothallic	Non papillate, persistent	*Quercus tarokoensis*		Taiwan	Jung *et al*., 2017a
2017	*P. mekongensis*	2	Sterile	Caducous	*Citrus grandis*	Confirmed	Vietnam	Crous *et al*., 2017
2017	*P. prodigiosa*	9	Sterile	Persistent	*Citrus grandis*	Confirmed	Vietnam	Puglisi *et al*., 2017
2017	*P. pseudopolonica*	6	Homothallic	Persistent	Water	Confirmed	China	Li *et al.,* 2017
2017	*P. tubulina*	12	Homothallic	Persistent/ caducous	*Fagus sylvatica*	Confirmed	Austria	Jung *et al*., 2017b
2017	*P. tyrrhenica*	7	Homothallic	Persistent	*Quercus ilex*	Confirmed	Italy	Jung *et al*., 2017b
2017	*P. versiformis*	12	Homothallic	Persistent	*Corymbia calophylla*	Confirmed	Australia	Paap *et al*., 2017
2017	*P. ×heterohybrida*	7	Self-sterile	Persistent	Water		Taiwan	Jung *et al*., 2017a
2017	*P. ×incrassata*	7	Self-sterile	Persistent	Water		Taiwan	Jung *et al*., 2017a

2017	*P. vulcanica*	7	Homothallic	Persistent	*F. sylvatica*	Confirmed	Italy	Jung *et al.*, 2017a
2018	*P. balyanboodja*	6	Sterile	Persistent	Soil		Australia	Burgess *et al*., 2018
2018	*P. betacei*	1	Heterothallic	Caducous	*Solanum betaceum*	Confirmed	Colombia	Mideros *et al*., 2018
2018	*P. cacuminis*	10	Sterile	Persistent	*Eucalyptus coccifera*		Australia	Khaliq *et al.,* 2018
2018	*P. condilina*	6	Homothallic	Persistent	*Casuarina obesa*		Australia	Burgess *et al.,* 2018
2018	*P. cooljarloo*	6	Homothallic	Persistent	*Hibbertia* sp.		Australia	Burgess *et al.,* 2018
2018	*P. kwongonina*	6	Homothallic	Persistent	*Banksia grandis*		Australia	Burgess *et al.,* 2018
2018	*P. oleae*	2	Homothallic	Persistent	*Olea europaea*	Confirmed	Italy	Ruano-Rosa *et al*., 2018
2018	*P. oreophila*	6	Homothallic	Persistent	Alpine herbfield		Australia	Khaliq *et al*., 2018
2018	*P. pseudorosacearum*	6	Homothallic	Persistent	*Persoonia longifolia*		Australia	Burgess *et al.,* 2018
2019	*P. abietivora*	7	Homothallic	Persistent	*Abies fraseri*	Confirmed	USA	Li *et al*., 2019
2019	*P. aleatoria*	1	Homothallic	Caducous/ persistent	*Pinus radiata*	Confirmed	New Zealand	Scott *et al.,* 2019b
2019	*P. chesapeakensis*	6	Sterile	Persistent	*Zostera marina*	Confirmed	USA	Man in 't Veld *et al*., 2019
2019	*P. urerae*	1	Heterothallic	Caducous	*Urera laciniata*	Confirmed	Perú	Grünwald *et al*., 2019
2020	*P. alpina*	1	Homothallic	Caducous	*Alnus viridis*	Confirmed	Italy	Bregant *et al*., 2020
2020	*P. aquae-coljargioo*	9	Homothallic	Persistent	Water		Australia	Crous *et al*., 2020a
2020	*P. aysenensis*	2	Homothallic	Persistent	*Aristotelia chilensis*	Confirmed	Chile	Crous *et al*., 2020b
2020	*P. personensis*	6	Sterile	Persistent	*Grevillea mccutcheonii*	Confirmed	Australia	Crous *et al*., 2020b
2021	*P. afrocarpa*	10	Sterile	Persistent	*Afrocarpus falcatus*		South Africa	Bose *et al*., 2021
2021	*P. cathayensis*	2	Homothallic	Persistent	*Carya cathayensis*	Confirmed	China	Morales-Rodríguez *et al.,* 2021
2021	*P. docyniae*	9	Sterile	Persistent	*Docynia indica*	Confirmed	Vietnam	Crous *et al*., 2021a
2021	*P. emzansi*	2	Homothallic	Persistent	*Afrocarpus falcatus*		South Africa	Bose *et al*., 2021
2021	*P. heterospora*	4	Heterothallic	Caducous	*O. europaea*	Confirmed	Italy	Scanu *et al.,* 2021
2021	*P. insulinativitatica*	2	Heterothallic	Caducous	Soil		Australia	Dang *et al*., 2021
2021	*P. kelmanii*	8	Heterothallic	Persistent	*Ptilotus pyramidatus*	Confirmed	Australia	Crous *et al*., 2021b
2021	*P. marrasii*	8	Homothallic	Persistent	*Cynara cardunculus*	Confirmed	Italy	Bregant *et al*., 2021a
2021	*P. mediterranea*	7	Heterothallic	Persistent	*Myrtus communis*	Confirmed	Italy	Bregant *et al*., 2021b
2021	*P. multibullata*	2	Heterothallic	Persistent	*Cinnamomum cassia*		Vietnam	Dang *et al*., 2021
2021	*P. theobromicola*	2	Heterothallic	Persistent	*Theobroma cacao*		Brazil	Decloquement *et al*., 2021
2021	*P. ×vanyenensis*	2	Heterothallic	Persistent	*Cinnamomum cassia*		Vietnam	Dang *et al*., 2021
2022	*P. celebensis*	10	Homothallic	Caducous	Water		Indonesia	Jung *et al.,* 2022
2022	*P. chilensis*	10	Homothallic	Caducous	Water		Chile	Jung *et al.,* 2022
2022	*P. javanensis*	10	Homothallic	Caducous	Water		Indonesia	Jung *et al.,* 2022
2022	*P. ludoviciana*	10	Sterile	Persistent	Water		USA	Jung *et al.,* 2022
2022	*P. multiglobulosa*	10	Homothallic	Caducous	Water		Indonesia	Jung *et al.,* 2022
2022	*P. novae-guineae*	5	Homothallic	Caducous	*Araucaria hunsteinii*		Papua New Guinea	Arentz, 2022
2022	*P. panamensis*	4	Homothallic	Persistent	Water		Panama	Chen *et al*., 2022

2022	*P. podocarpi*	n/d	Homothallic	Persistent	*Podocarpus totara*	Confirmed	New Zealand	Dobbie *et al.*, 2022
2022	*P. procera*	10	Sterile	Persistent	Water		USA	Jung *et al.*, 2022
2022	*P. pseudochilensis*	10	Homothallic	Caducous	Water		Chile	Jung *et al.*, 2022
2022	*P. pseudogallica*	10	Sterile	Persistent	Water		Vietnam	Jung *et al.*, 2022
2022	*P. pseudokernoviae*	10	Homothallic	Caducous	Water		Chile	Jung *et al.*, 2022
2022	*P. scandinavica*	10	Homothallic	Persistent	Water		Sweden	Jung *et al.*, 2022
2022	*P. subarctica*	10	Sterile	Persistent	Water		Sweden	Jung *et al.*, 2022
2022	*P. tenuimura*	10	Homothallic	Persistent	Water		USA	Jung *et al.*, 2022
2022	*P. tonkinensis*	10	Homothallic	Persistent	Water		Vietnam	Jung *et al.*, 2022
2022	*P. transitoria*	3	Sterile	Persistent	*Q. robur*		Czech Republic	Chen *et al*., 2022
2022	*P. ukrainensis*	10	Sterile	Persistent	Water		Ukraine	Jung *et al.*, 2022
2022	*P. variabilis*	7	Homothallic	Persistent	Water		Slovakia	Chen *et al*., 2022
2022	*P. viadrina*	6	Homothallic	Persistent	*Q. robur*		Poland	Tan *et al*., 2022

The geographical distribution of the species described by clade varies considerably according to adaptation, growth temperature of the various species and range of the host plants. The number is also strongly influenced by the greater presence of research groups on this topic in the US, Europe and Oceania than other continents.

Some evolutionary clades such as 1, 2, 7, 9 and 10 show a rather wide worldwide distribution in different continents, while others appear much more geographically limited. Clades 4 and 5 appear to be typical of tropical areas and the southern hemisphere, whereas clade 3 in colder areas of the northern hemisphere (Hansen *et al*., 2017). Little is known about clade 11, with only one species (*Phytophthora lilii*) described in Japan (Rahman et *al.,* 2015) (Fig. 2).

Despite the research conducted so far, the number of known species probably does not reflect the real biodiversity of this genus. Recent studies estimate that at least another 300-500 species will be discovered in unexplored forests and natural ecosystems (Brasier, 2009; Scott *et al*., 2019a).

In addition to the formally described species, a *nomen nudum* has been assigned to a large number of *Phytophthora* species (over 30) and are pending a formal description. Some of these species are very important pathogens with a large geographic distribution and host range. Extensive studies on morphology and taxonomy have recently allowed the name of some of these species to be stabilized: notable examples are *Phytophthora bilorbang*, *P. chlamydospora*, *P. emzansi*, *P. lacustris* and *P. kelmanii*, previously reported as *Phytophthora* taxon oaksoil, *P.* taxon Pg chlamydo, *P.* taxon emzansi, *P.* taxon salixsoil and *P.* taxon kelmania respectively (Aghighi *et al*., 2012; Nechwatal *et al*., 2013; Hansen *et al*., 2015; Crous *et al*., 2021b).

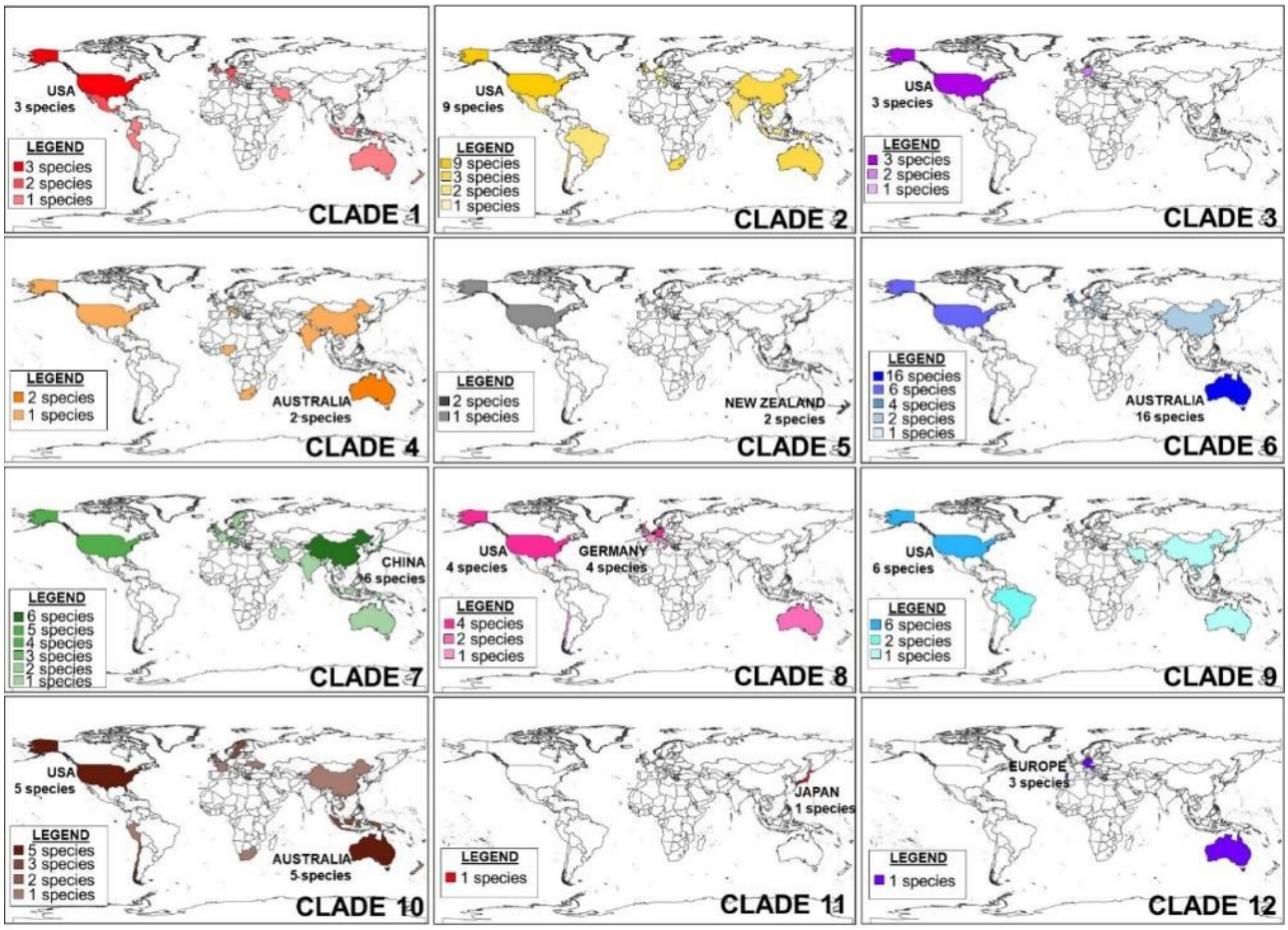

Figure 2. Geographical distribution of *Phytophthora* species belonging to the twelve phylogenetical clades per country of description.

Most *Phytophthora* species are polyphagous pathogens, for example *P. cactorum* and *P. cinnamomi* have a very broad host and geographic range, infecting more than 5000 woody and herbaceous, dicotyledonous, monocotyledonous and coniferous plant species. Whereas only a few species seem to be host specific, such as *P. quercina* on *Quercus* spp., *P. castanetorum* on *Castanea sativa*, *P. ilicis* on *Ilex aquifolium* and *P. oleae* on olive (Jung *et al*., 1999, 2017b; Bertier *et al.,* 2013; Scanu *et al*., 2014b; Ruano-Rosa *et al*., 2018). Moreover, many species of phylogenetic clades 6, 9 and 10 exhibit a saprophytic and/or opportunistic behaviour, living mainly in aquatic habitats such as rivers, streams or the irrigation water in nurseries and contributing to organic decomposition (Brasier *et al*., 2003b; Marano *et al*., 2016).

The diffusion of *Phytophthora* species is a critical challenge not only for natural ecosystems but also for the horticulture industry and reforestation and restoration programmes. Nurseries represent an optimal environment for *Phytophthora* owing to the occurrence of various plant species in a limited space, often imported from different geographical areas (Antonelli *et al*., 2022). These conditions are

ideal for host jumps by pathogens, often vehiculated by splashing water drops and with irrigation water (Moralejo *et al*., 2009; Parke *et al*., 2019; Redekar *et al*., 2019; Antonelli *et al*., 2022). At the same time, the intensive use of chemicals in a nursery can promote the formation of fungicide-resistance in pathogen populations (Bruin & Edgington, 1981; Lukas *et al*., 1990; Dobrowolski *et al*., 2008). In addition to the direct economic losses caused to the horticulture industry, *Phytophthora* spp. pose a threat to productivity of reforestation and restoration sites (Webber *et al*., 2010; Prospero *et al*., 2013; Simamora *et al.,* 2018; Frankel *et al*., 2020).

Another problem is related to the potential ability of these microorganisms to hybridize in an interspecific way due to the lack of genetic barriers between zoospores of different species (Brasier *et al*., 2004). Therefore, the meeting of endemic with exotic species in a nursery habitat can easily form new and dangerous *Phytophthora* hybrids that could pose a serious threat to both agricultural and forest ecosystems (Brasier *et al*., 2004; Man in't Veld *et al*., 2007, 2012).

Over the last three decades an increasing number of emerging diseases caused by *Phytophthora* species has been recognised in natural and agriculture systems worldwide (Jung *et al*., 2018; Scott *et al.,* 2019a; Linaldeddu *et al*. 2023). The most notable *Phytophthora* outbreaks in natural ecosystems that have occurred recently include the 'Sudden Oak Death' and 'Sudden Larch Death' caused by *P. ramorum* in the western USA and UK (Rizzo *et al.,* 2002; Brasier & Webber, 2010), the mortality of *Austrocedrus chilensis* in Patagonia due to *P. austrocedrae (*Greslebin *et al*., 2007), the alder decline in the north hemisphere caused by multiple *Phytophthora* species (Jung & Blansche, 2004; Sims *et al*., 2015; Bregant *et al*., 2020), the extensive dieback of natural primary forests in New Zealand caused by *P. agathidicida* (Beever *et al.,* 2009; Weir *et al*., 2015) and the decline of Mediterranean vegetation in Italy (Linaldeddu *et al*., 2014; Scanu *et al*., 2015). Although the primary action of a few species has been ascertained in the aetiology of many diseases, recent studies have highlighted that multiple *Phytophthora* species can interact in the pathogenesis process. Until a few years ago, the diseases were associated with a single pathogenic species; however, recent studies have demonstrated a complex aetiology in which several *Phytophthora* species have a seasonal and synergistic activity (Bregant *et al*., 2020; Benigno *et al*., 2023; Linaldeddu *et al*., 2023).

Phytophthora cinnamomi, for example*,* is considered the main pathogen involved in oak decline in Europe (Brasier *et al.,* 1993; Jung *et al.*, 1999; Vettraino *et al.,* 2002, Perez-Sierra *et al*., 2013; Linaldeddu *et al*., 2014). However, extensive surveys recently conducted in oak formations emphasize the occurrence of several other species associated with symptomatic oak trees (Linaldeddu *et al*., 2014; Jung *et al*., 2018; Seddaiu *et al*., 2020). In particular, *Phytophthora quercina*, *P. gonapodyides* and *P. plurivora* are considered the most common species involved in the aetiology of root and collar rot on oaks (Fig. 3).

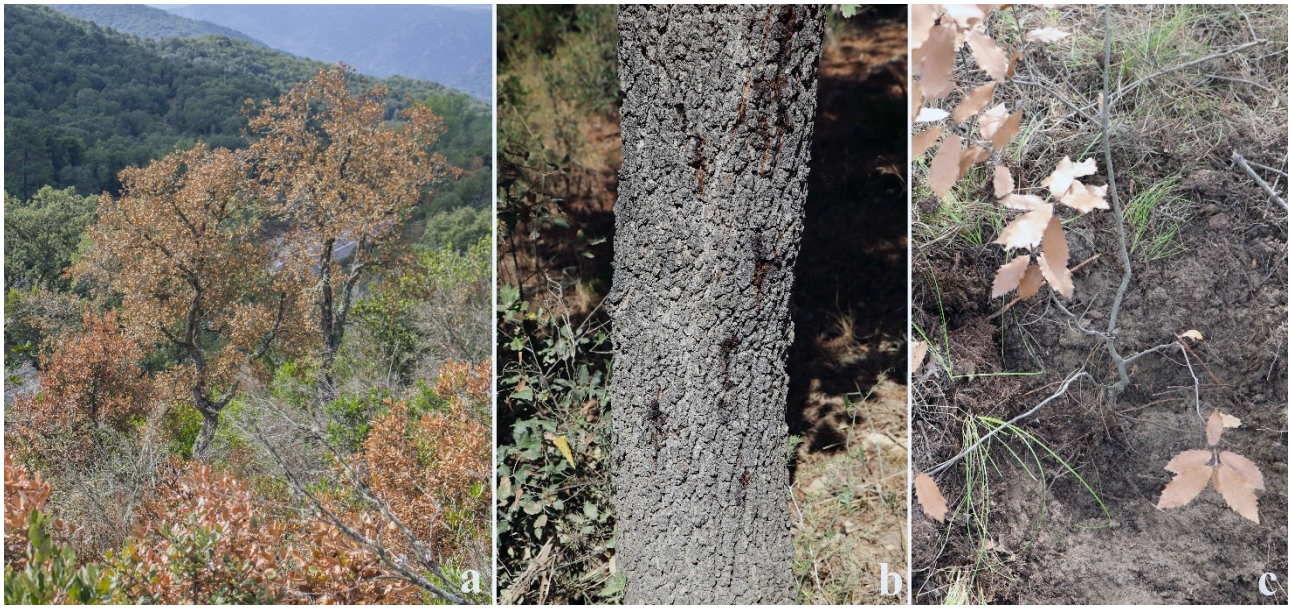

Figure 3. Overview of *Phytophthora* related-disease symptoms on oaks: sudden death (a), bleeding cankers (b) and root rot (c).

Aim of the thesis

Recently, serious outbreaks of *Phytophthora* diseases have been detected in natural woodlands in Italy, chiefly in Mediterranean ecosystems (Picco *et al*., 2011; Linaldeddu *et al*., 2014; Scanu *et al*., 2015; Benigno *et al*., 2023). Out of the 220 *Phytophthora* species described worldwide, 65 species are currently reported in Italy associated with various disease symptoms on root, crown, stem, bud, fruit and leaves of alders, ash, chestnut, juniper, lentisk and oaks (Vettraino *et al.*, 2002, 2005; Scanu *et al*., 2015; Bregant *et al*., 2020; Benigno *et al*., 2023).

Despite the large number of studies conducted on *Phytophthora*-related diseases in natural ecosystems in Italy, little is still known about the real distribution, diversity and impacts of these pathogens in mountain ecosystems, including the Alps. A recent field survey conducted in the north-eastern Italian Alps has shown the occurrence of severe *Phytophthora* outbreaks on many tree species typical of mountain habitats such as alders (Bregant *et al*., 2020). This study has permitted a new species to be discovered and described, namely *Phytophthora alpina* and more generally to discover a great biodiversity of species able to grow at low temperatures (Bregant *et al*., 2020).

Therefore, given this alarming scenario on the spread of *Phytophthora* outbreaks in mountain areas and the still limited information on the species involved, a study was conducted across different types of mountain vegetation in Italy, Portugal and Slovenia to isolate and characterize the main pathogens associated. At the same time the presence of *Phytophthora* in nurseries was evaluated.

The methodologies used and the results obtained in the investigations conducted for this thesis are presented in the next four chapters that reflect four scientific articles, three of which have already been published in authoritative scientific journals whereas one is still under review.

Chapter I

Diversity of *Phytophthora* species involved in new diseases of mountain vegetation in Europe with the description of *Phytophthora pseudogregata* sp. nov.

Reprinted from: Bregant C., Rossetto G., Meli L., Sasso N., Montecchio L., Brglez A., Piškur B., Ogris N., Maddau L., Linaldeddu B.T. 2023. Diversity of *Phytophthora* species involved in new diseases of mountain vegetation in Europe with the description of *Phytophthora pseudogregata* sp. nov. *Forests* 14(8): 1515.

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Diversity of *Phytophthora* species involved in new diseases of mountain vegetation in Europe with the description of *Phytophthora pseudogregata* sp. nov.

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Citation: Bregant, C.; Rossetto, G.; Meli, L.; Sasso, N.; Montecchio, L.; Brglez, A.; Piškur, B.; Ogris, N.; Maddau, L.; Linaldeddu, B.T. Diversity of *Phytophthora* Species Involved in New Diseases of Mountain Vegetation in Europe with the Description of *Phytophthora pseudogregata* sp. nov. *Forests* **2023**, *14*, 1515. https://doi.org/10.3390/f14081515

Academic Editors: Salvatore Moricca and Tiziana Panzavolta

Received: 29 June 2023
Revised: 23 July 2023
Accepted: 24 July 2023
Published: 25 July 2023

Abstract: New and emerging *Phytophthora*-related diseases in small trees, shrubs and herbaceous plants typical of subalpine vegetation have recently been observed in Italy and Slovenia. Diseased plants showed a complex symptomatology including foliar necrosis, fruit rot, shoot blight and branch bleeding cankers. Since little information is available about the aetiology of these aerial *Phytophthora* diseases, from 2019 to 2022, field surveys were conducted in 54 sites to define the occurrence, distribution and impact of the *Phytophthora* species on mountain vegetation. A total of 360 *Phytophthora* isolates were obtained from 397 samples collected from 33 herbaceous and woody host species. Based on phylogenetic analysis and morphometric data, 17 *Phytophthora* species were identified: *P. pseudosyringae* (201 isolates), *P. plurivora* (54), *P. gonapodyides* (21), *P. ilicis* (20), *P. alpina* (17), *P. acerina* (11), *P. cactorum* (7), *P. pseudocryptogea* (6), *P. cambivora* (5), *P. idaei* (4), *P. psychrophila* (3), *P. bilorbang* (2), *P. chlamydospora* (2), *P. hedraiandra* (1), *P. kelmanii* (1), *P. rosacearum* (1) and *P. syringae* (1). In addition, three isolates of a new putative *Phytophthora* species obtained from *Alnus viridis*, *Juniperus communis* and *Rhododendron ferrugineum* are described here as *Phytophthora pseudogregata* sp. nov. Overall, the results highlighted an unexpectedly high diversity of *Phytophthora* species in mountain areas, with many species able to cause aerial infections due to the production of caducous sporangia.

Keywords: alpine regions; emerging disease; oomycetes; foliar necrosis; ITS clade 6; phylogeny

1. Introduction

The large genus *Phytophthora* de Bary includes several invasive plant pathogens that represent an increasing threat to forest ecosystems and agriculture productions worldwide [**1–4**]. Over the last 20 years, scientific

interest in this group of oomycetes has increased rapidly in forest pathology and this has led to the discovery of several new species and pathosystems [**5–8**].

Most of the known *Phytophthora* species have a soilborne or waterborne lifestyle, due to the production of persistent sporangia and the release of motile zoospores [**9**,**10**]. The majority of *Phytophthora* species are necrotrophic or hemibiotrophic pathogens, able to cause root rot diseases in herbaceous and woody plant hosts; whereas a few species, especially those strongly associated with water habitats, can also survive as saprophytes [**11**]. The main symptoms caused by pathogenic *Phytophthora* species with a soilborne lifestyle include fine root losses, root rot, collar necrosis and stem bleeding cankers. Plants with root and collar infections show nonspecific secondary symptoms at the canopy level, such as epicormic shoots and sudden death [**1**,**12**].

Conversely, *Phytophthora* species with an airborne or mixed airborne and soilborne lifestyle have the ability to produce caducous sporangia and infect fruits, leaves, shoots, twigs and branches, causing necrosis, rots and an anticipated loss of organs [**1**,**13–15**]. Caducous sporangia can act directly as infective propagules or release motile zoospores [**1**]. Aerial *Phytophthora* infection can occur actively via lenticels or stomata in the epigeal organs of the host [16]. The ability to produce caducous sporangia is a feature common in the species belonging to clades 1, 3, 4 and 8 [**17**]. Within this last clade, one of the most aggressive species is *Phytophthora ramorum*, known to cause leaf blight, shoot blight and bleeding cankers on forest and ornamental plant species in the temperate areas of North America and Europe [**5**,**18**,**19**]. Other species belonging to clade 8, such as *P. foliorum* and *P. hibernalis* have been reported as airborne pathogens on *Rhododendron* and *Citrus* spp. [**20–22**]. In agriculture and horticulture, species of clades 1 and 4, such as *P. cactorum*, *P. infestans*, *P. nicotianae* and *P. palmivora*, are well known to cause leaf, stem and fruit diseases on many herbaceous and wood crops [**23–29**].

Clade 3 includes a few cryptic species characterized by a partial aerial lifestyle with a relatively low optimum temperature for growth and a common association with native forest species [**14**,**30**,**31**]. In particular, *Phytophthora pseudosyringae* is emerging as an invasive pathogen on a broad number of hosts at global scale [**14**,**32–34**].

In Europe, aerial *Phytophthora* diseases have been studied mainly on agricultural crops [**1**,**26**,**35**,**36**,**37**,**38**] and to a much lesser extent on forest trees, especially in subalpine ecosystems [**14**]. Alpine and subalpine regions are important biodiversity hotspots for the flora, including a large number of plants and many endemisms in very confined environments and extreme conditions [**39**]. Due to the huge floristic diversity in small spatial scales, mountain forests could represent useful models to understand the ecological and evolutionary host–pathogen dynamics and to conserve pristine ecosystems [**40**,**41**].

Therefore, given the growing expansion of *Phytophthora* diseases in subalpine ecosystems in Italy and Slovenia and the still limited information available about these pathosystems, a study was conducted to isolate, identify and characterize the main pathogens associated with these new and emerging diseases.

2. Materials and Methods

2.1. Field Survey and Sampling Procedure

Field surveys were conducted from autumn 2019 to summer 2022 in 54 sites distributed in different mountainous areas of Northeast Italy, Sardinia and Western Slovenia (Table 1). The monitored sites are located at an altitude ranging from the valley bottom (700 m a.s.l.) to above the tree line (2100 m a.s.l.) and

include forests, riparian ecosystems and heathlands typical of alpine and subalpine formations.

At each site, plants were visually checked for the occurrence of typical *Phytophthora* symptoms, such as leaf and fruit necrosis, shoot blight, wilting twigs, branches dieback and bleeding cankers. In the most impacted formations, the disease incidence and mortality rate were estimated along 25 m long linear plots. Disease incidence was calculated as the number of symptomatic trees out of the total number of trees (DI = n/N × 100) and mortality as the number of dead trees out of the total number of trees (M = d/N × 100) [**42**].

In each site, a variable number of tissue samples of leaves, twigs and branches was collected from symptomatic plants (Table 1). Overall, 397 samples were collected from 33 host species, small trees, shrubs and herbaceous plants. These included *Acer monspessulanum*, *Acer pseudoplatanus*, *Alnus cordata*, *Alnus glutinosa*, *Alnus incana*, *Alnus viridis*, *Betula pubescens*, *Calluna vulgaris, Erica carnea, Fagus sylvatica*, *Fragaria vesca*, *Fraxinus excelsior, Genista corsica*, *Ilex aquifolium*, *Juniperus communis*, *Laburnum alpinum*, *Larix decidua*, *Lonicera alpigena*, *Lycopodium clavatum*, *Pinus mugo*, *Populus tremula*, *Quercus pubescens*, *Rhododendron ferrugineum*, *Rhododendron hirsutum*, *Rubus idaeus*, *Salix alpina, Salix atrocinerea*, *Salix caprea*, *Sorbus aria, Sorbus aucuparia, Taxus baccata, Vaccinium myrtillus* and *Vaccinium vitis-idaea (*Table 1). The samples were sealed in plastic bags, labelled and used for *Phytophthora* isolations within 24–48 h.

Table 1. Study sites information, plant species monitored and number of samples collected.

Survey Sites	Country	Elevation (m a.s.l.)	Geographic Coordinates		Sampled Species*
1	Italy	1030	46.4711671	12.4611700	Sau(3), Vm(5), Pt(4), La(2), Fe(2), Fv(2)
2	Italy	912	46.4622684	12.4746272	Ai(2), Sc(1)
3	Italy	1220	46.4675090	12.4833650	Fe(2), Lc(12), Fv(1)
4	Italy	1060	46.4729600	12.4668290	Vm(11), Pt(6), Fe(2), Sc(1)
5	Italy	1012	46.4798320	12.5178940	Fe(2), Vm(2)
6	Italy	1900	46.4498739	12.5011762	Rf(2)
7	Italy	1692	46.4491930	12.5041780	Pm(2), Vm(1)
8	Italy	1757	46.4777890	12.5932750	Av(18), Ri(4), Pm(1)
9	Italy	1691	46.4760760	12.6366770	Av(3)
10	Italy	1251	46.4926832	12.5620431	Ai(9)
11	Italy	1841	46.4852460	12.5585580	Av(2)
12	Italy	1912	46.4789100	12.5493510	Ld(1), Sau(1)
13	Italy	1752	46.5811670	12.2562680	Vm(6), Jca(3), Rf(1), Vv(1), La(1)
14	Italy	1866	46.5962680	12.2694770	Jc(2), Vm(2), Pm(1)
15	Italy	1947	46.5109880	12.3933840	Pm(5), Vm(2), Jca(1)
16	Italy	1566	46.4052340	12.4649810	Vm(3), Bp(2), Sa(1), Pm(1)
17	Italy	1725	46.6486230	12.4474240	Av(2), Sc(1)
18	Italy	1860	46.6663880	12.4912310	Vm(8), Rf(3)
19	Italy	1882	46.6644254	12.4493550	Vm(11), Vv(2), Jc(2), Cv(2), Rf(2), Pm(1), Ec(2)
20	Italy	1920	46.6008270	12.5590990	Vm(12), Jc(7), Cv(2), Rf(6), Rh(2)
21	Italy	1603	46.6025622	12.5915985	Jc(1)
22	Italy	1320	46.5898670	12.5807640	Pt(10)
23	Italy	1796	46.4067140	12.0764750	Sc(1), Av(4), Pm(2), Jc(3)
24	Italy	1074	46.0994836	46.0994836	Fe(2)
25	Italy	1550	45.9477727	12.0087351	Jc(3)
26	Italy	1670	45.9746300	11.4080100	Jc(10), Sc(2)
27	Italy	1273	45.9428700	11.4237400	Sc(6)
28	Italy	1199	45.9412300	11.4330700	Sc(2)
29	Italy	1009	45.8648319	11.5232058	Fe(1)

30	Italy	1337	45.8462570	11.7940760	Jc(4), Sar(2)
31	Italy	1760	46.3797890	13.4884770	Rf(2), Sc(2), Sa(1)
32	Italy	1355	46.3787840	13.4757220	Sa(2), Pm(1)
33	Italy	888	46.5059780	13.2630950	Ai(1)
34	Italy	1633	46.2133271	13.5278655	Sau(2), Sc(1)
35	Italy	1830	46.5753410	13.1770300	Vm(5), Rf(4), Av(2), Vv(1)
36	Italy	1990	46.5705310	13.0514810	Av(2)
37	Italy	1010	46.5379836	13.0856086	Fe(1)
38	Italy	1735	46.1515890	11.5346470	Vm(13), Rf(6)
39	Italy	1750	46.1376260	11.5419110	Vm(6), Av(1), Pt(3)
40	Italy	2065	46.8869230	12.2004166	Vm(6), Rf(4), Jc(1), Av(1)
41	Italy	1502	46.6554920	12.3509230	Vv(2), Rf(1), Pm(1)
42	Italy (Sardinia)	1125	40.0437071	9.2064231	Ia(2), Ag(3), Am(2), Qp(4)
43	Italy (Sardinia)	1328	40.0326060	9.2456210	Ia(1), Sat(1)
44	Italy (Sardinia)	1517	40.0177590	9.2788890	Jc(5), Gc(2), Ag(1)
45	Italy (Sardinia)	860	39.9386700	9.4810890	Ac(3)
46	Italy (Sardinia)	980	40.3495542	8.8807715	Ia(14)
47	Italy (Sardinia)	1029	40.4227424	8.9957414	Ia(6), Tb(1)
48	Italy (Sardinia)	825	39.9213336	9.4757911	Ag(2)
49	Slovenia	1755	46.3549947	13.9057009	Av(2), Pm(1), Ld(1), Vm(1), Sc(1), Ap(1), Sau(1)
50	Slovenia	1615	46.2378426	13.9943075	Av(3), Sar(1), Rf(1), Sc(2), Vm(1), Fs(1)
51	Slovenia	704	46.2732634	13.9879130	Ai(2)
52	Slovenia	1450	45.9788312	13.8629901	Fs(1)
53	Slovenia	1495	45.9785910	13.8643530	Pm(2)
54	Slovenia	907	45.9378821	13.9793240	Fs(1), Ap(1)

* In brackets the number of samples collected from each plant species: *Acer monspessulanum* (Am), *Acer pseudoplatanus* (Ap), *Alnus cordata* (Ac), *Alnus glutinosa* (Ag), *Alnus incana* (Ai), *Alnus viridis* (Av), *Betula pubescens* (Bp), *Calluna vulgaris* (Cv), *Erica carnea* (Ec), *Fagus sylvatica* (Fs), *Fragaria vesca* (Fv), *Fraxinus excelsior* (Fe), *Genista corsica* (Gc), *Ilex aquifolium* (Ia), *Juniperus communis* (Jc), *Laburnum alpinum* (La), *Larix decidua* (Ld), *Lonicera alpigena* (La), *Lycopodium clavatum* (Lc), *Pinus mugo* (Pm), *Populus tremula* (Pt), *Quercus pubescens* (Qp), *Rhododendron ferrugineum* (Rf), *Rhododendron hirsutum* (Rh), *Rubus idaeus* (Ri), *Salix alpina* (Sa), *Salix atrocinerea* (Sat), *Salix caprea* (Sc), *Sorbus aria* (Sar), *Sorbus aucuparia* (Sau), *Taxus baccata* (Tb), *Vaccinium myrtillus* (Vm) and *Vaccinium vitis-idaea* (Vv).

2.2. Phytophthora Isolation and Characterization

Phytophthora isolation was performed directly from the symptomatic tissue samples. Necrotic leaves were externally disinfected and cut in small pieces along the border of active lesions, whereas shoots and bark samples from bleeding cankers, after removing the outer bark, were cut in small fragments (along the margin of each lesion) with a sterile scalpel. In both cases, small pieces of 3–5 mm^2 were placed on 90 mm diameter Petri dishes containing the selective medium PDA+ [**14**]. In samples that resulted negative, the procedure was repeated up to three times. After incubation at 20 °C for 3 days in the dark, hyphal tips of emerging colonies were taken and transferred into new PDA and carrot agar (CA) Petri dishes and incubated at 20 °C in the dark.

Isolates were morphologically examined and then grouped into morphotypes based on colony appearance and morpho-biometric data of sporangia, oogonia, chlamydospores and hyphal swellings. To enhance the production of sporangia, CA plugs of each isolate were placed in Petri dishes containing pond water and asymptomatic alder roots. Petri dishes were kept at 20 °C in the dark and sporangia production was assessed every 12 h for 3 days.

For the new putative species, colony morphology was determined on 7-day-old cultures incubated at 20 °C in the dark as reported in Bregant *et al*. [**14**]. Cardinal temperatures for growth were evaluated on CA plates incubated at 2,

5, 10, 15,18, 20, 23, 25, 27, 30, 32 and 34 °C (±0.5 °C) in the dark. Five replicates for each isolate were made and colony diameter was measured after 7 days. Morphology of sporangia (n. 50) and the ability to produce hyphal swellings and chlamydospores was recorded for each isolate. Breeding system was examined after 20 days on CA at 20 °C in the dark. Measurements and photos of the morphological structures (sporangia, chlamydospores, hyphal swellings, oogonia and anteridia) were recorded using the software Motic Images Plus 3.0 paired with a Moticam 10+ camera connected to a Motic BA410E microscope. The sizes are presented as mean values ± standard deviation.

Representative isolates of each species were stored on PDA and CA slants under oil in the culture collection of the Dipartimento Territorio e Sistemi Agro-Forestali, Università degli Studi di Padova.

The ex-type culture of the new species was deposited at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, and nomenclatural data in MycoBank (www.MycoBank.org, accessed on 29 June 2023). The holotype was lodged with the herbarium of Westerdijk Fungal Biodiversity Institute as a dried culture on CA.

2.3. Molecular Identification of the Isolates

Isolation of genomic DNA was performed from the mycelium of 7-day-old *Phytophthora* colonies as reported in Linaldeddu *et al*. [**28**]. For all isolates, the internal transcribed spacer (ITS) region of the rDNA, including the 5.8S rRNA gene, was amplified and sequenced using the universal primers ITS1 and ITS4 [**43**]. ITS sequences were used to confirm the identification at species level. For three isolates of the new putative species another two DNA regions, namely ß-tubulin (Btub) and cytochrome c oxidase subunit I (*cox*1), were amplified and sequenced using the primer-pairs TUBUF2/TUBUR1 and FM84/FM83 [**44**,**45**], respectively. Polymerase chain reactions (PCR) were performed in 50 µl reaction mixtures using the GoTaq® Hot Start Green Master Mix (Promega, Milano, Italy) and a SimpliAmp Thermal Cycler (Thermo Fisher Scientific Inc, Waltham, Massachusetts, US). Amplification conditions for the three DNA regions were as follows: an initial denaturation step at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final elongation step of 7 min at 72 °C for ITS; an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 9;°C for 40 s, annealing at 54 °C for 1 min, extension at 72 °C for 1 min and a final elongation step of 7 min at 72 °C for Btub; and an initial denaturation at 95 °C for 2 min followed by 38 cycles at 95 °C for 25 s, 53 °C for 50 s, 72 °C for 70 s and a final extension step of 9 min at 72 °C for *cox1*.

The PCR products were purified using the Monarch™ PCR & DNA Cleanup Kit according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA) and sequenced by BMR Genomics DNA sequencing service (www.bmr-genomics.it). Sequences were edited with FinchTV v1.4.0 (Geospiza, Inc., http://www.geospiza.com/finchtv) and compared with sequences of ex-type culture deposited in GenBank (http://blast.ncbi.nlm.nih.gov). New sequences were deposited in GenBank (Table 2).

Table 2. Details of *Phytophthora* isolates included in the phylogenetic analyses. Ex-type cultures are given in bold typeface and newly generated sequences are indicated in italics.

Species	Collection No.	Host	GenBank Accession Number		
			ITS	Btub	*Cox*1
Phytophthora acerina	**CBS 133931**	*Acer pseudoplatanus*	JX951285	-	-
P. acerina	CB222	*Juniperus communis*	*OR167204*	-	-
P. agathidicida	**P15175**	*Agathis australis*	KP295308	-	-

P. alpina	**CBS 146801**	*Alnus viridis*	MT707332	-	-
P. alpina	CB387	*Vaccinium myrtillus*	*OR167205*	-	-
P. alticola	**TBF0060A10**	*Eucalyptus grandis*	KX247599	-	-
P. amnicola	**CBS 131652**	Water	JQ029956	JQ029952	MH477740
P. amnicola	VHS 19503	Water	JQ029958	JQ029954	JQ029950
P. asparagi	**VHS 17644**	*Lomandra sonderi*	EU301168	JN547592	HQ012845
P. austrocedrae	**CBS 122.911**	*Austrocedrus chilensis*	DQ995184	-	-
P. bilorbang	**CBS 161653**	*Rubus anglicandicans*	JQ256377	JQ256374	MH477742
P. bilorbang	SA146	*R. anglicandicans*	JN547624	JN547585	JN547646
P. bilorbang	CB600	*J. communis*	*OR167206*	-	-
P. borealis	**CBS 132023**	Water	HM004232	JQ626615	MH136854
P. borealis	AKWA 57.2-0708	Water	JQ626598	JQ626614	JQ626624
P. cactorum	**CBS 231.30**	*Syringa vulgaris*	MG783385	-	-
P. cactorum	CB389	*Sorbus aria*	*OR167207*	-	-
P. cambivora	**CBS 114087**	*Castanea sativa*	MG783387	MH493913	MH136860
P. cambivora	CB400	*Alnus incana*	*OR167208*	-	-
P. captiosa	**CBS 119107**	*Eucalyptus* sp.	DQ297402		
P. castaneae	**ICMP 19434**	*Castanea crenata*	KP295319	-	-
P. castanetorum	**CBS 142299**	*C. sativa*	MF036182	-	-
P. chlamydospora	**P6133**	*Prunus* sp.	MG865471	MH493919	MH136867
P. chlamydospora	VHS 3753	Soil	EU301160	JN547616	HQ012878
P. chlamydospora	CB480	*Salix alpina*	*OR167209*	-	-
P. cinnamomi	**CBS 144.22**	*Cinnamomum burmannii*	MG865473	-	-
P. citrophthora	**CBS 950.87**	*Citrus* sp.	MG865476	-	-
P. clandestina	**CBS 347.86**	*Trifolium subterraneum*	MG865477	-	-
P. cocois	**P19948**	*Cocos nucifera*	KP295304	-	-
P. crassamura	**PH138**	*Juniperus phoenicea*	KP863493	KX251202	KP863485
P. crassamura	CB267	*Cynara cardunculus*	MZ569853	OQ067252	OQ067256
P. dauci	**CBS 127102**	*Daucus carota*	KC478761	-	-
P. elongata	**CBS 125799**	*Eucalyptus marginata*	GQ847754	-	-
P. fluvialis	**CBS 129424**	Water	MG865491	JN547595	MH136887
P. fluvialis	VHS 17350	Water	EU593261	JN547593	JF701440
P. fragariaefolia	**CBS 135747**	*Fragaria* × *ananassa*	AB819580	-	-
P. gibbosa	**CBS 127951**	*Acacia pycnantha*	MG865499	MH493942	MH136894
P. gibbosa	VHS 22008	*Grevillea* sp.	HQ012936	JN547597	HQ012849
P. gonapodyides	**P7050**	*Alnus* sp.	MG865501	MH493944	MH136896
P. gonapodyides	SLPA72	*Eucalyptus obliqua*	HQ012937	JN547598	HQ012850
P. gonapodyides	CB367	*J. communis*	*OR167210*	-	-
P. gregata	**CBS 127952**	*Patersonia* sp.	MG865503	MH493945	MH477746
P. gregata	MJSP235	*Pinus radiata*	EU301172	JN547602	HQ012853
P. hedraiandra	**CBS 111725**	*Viburnum* sp.	MG865504	-	-
P. hedraiandra	CB415	*A. viridis*	*OR167211*	-	-
P. hydrogena	**P19968**	Water	KC249959	-	-
P. idaei	**CBS 971.95**	*Rubus idaeus*	MG865509	-	-
P. idaei	CB101	*R. idaeus*	*OR167212*	-	-
P. ilicis	**P3939**	*Ilex aquifolium*	MG865511	-	-
P. ilicis	CB265	*I. aquifolium*	*OR167213*	-	-
P. inundata	**CBS 216.85**	*Salix matsudana*	MG865516	MH493958	MH136910
P. ipomoeae	**CBS 109229**	*Ipomoea longipedunculata*	KF777191	-	-
P. irrigata	**P16861**	Water	MG865520		
P. kelmanii	**CBS 146551**	*Ptilotus pyramidatus*	MT210487	-	-
P. kelmanii	**CB426**	*A. incana*	*OR167214*		
P. lacustris	**P245**	*S. matsundana*	JQ626605	JQ626619	MH136916
P. lacustris	HSA1959	Water	HQ012956	JN547618	HQ012880
P. lilii	**CBS 135746**	*Lilium longiflorum*	MG865523	-	-
P. litoralis	**CBS 127953**	*Banksia* sp.	MG865526	MH493967	MH136921
P. litoralis	VHS 19173	*Banksia* sp.	EU869199	JN547610	HQ012865

P. marrasii	**CBS 148033**	*Cynara cardunculus*	MZ569854	-	-
P. megakarya	**CBS 238.83**	*Theobroma cacao*	HQ261610	-	-
P. megasperma	**CBS 402.72**	n/d	MG865535	MH493973	MH136930
P. megasperma	ME16	*Punica granatum*	OP999676	OQ067253	OQ067257
P. mississippiae	**P19994**	Water	MG865542	MH493980	MH136935
P. mississippiae	57J4	Water	KX251313	KF112853	KF112861
P. moyootj	**CBS 138659**	Soil	KJ372256	KJ372303	MH477750
P. moyootj	DH103	Water	KJ372255	KJ372301	KJ396700
P. niederhauserii	**P10616**	*Hedera helix*	AY550915	-	-
P. ornamentata	**CBS 140647**	*Pistacia lentiscus*	MG865556	MN207275	MH136947
P. ornamentata	PH153	*P. lentiscus*	KP863497	MN207276	KP863487
P. palmivora	**CBS 305.62**	*Areca catechu*	MG865559	-	-
P. pinifolia	**CBS 122924**	*Pinus radiata*	MG865566	MH493999	MH136958
P. pinifolia	CMW 26669	*P. radiata*	EU725807	JN935979	JN935961
P. plurivora	**CBS 124093**	*Fagus sylvatica*	MG865568	-	-
P. plurivora	CB358	*J. communis*	*OR167215*	-	-
P. polonica	**P131445**	*A. glutinosa*	DQ396410	-	-
P. pseudocryptogea	**CBS 139749**	*Isopogon buxifolius*	KP288376	-	-
P. pseudocryptogea	CB482	*J. communis*	*OR167216*	-	-
P. pseudogregata	**CBS 149859**	*J. communis*	*OR167217*	*OR189513*	*OR189516*
P. pseudogregata	CB308	*Rhododendron ferrugineum*	*OR167218*	*OR189514*	*OR189517*
P. pseudogregata	CB366	*Alnus viridis*	*OR167219*	*OR189515*	*OR189518*
P. pseudosyringae	**CBS 111772**	*Quercus robur*	MG865574	-	-
P. pseudosyringae	CB303	*J. communis*	*OR167220*	-	-
P. psychrophila	**CBS 803.95**	*Q. robur*	MG865576	-	-
P. psychrophila	CB195	*Quercus pubescens*	*OR167221*	-	-
P. quercina	**CBS 784.95**	*Quercus* sp.	MG865578	-	-
P. quininea	**CBS 407.48**	*Cinchonae officinalis*	MG865580	-	-
P. ramorum	**CBS 101553**	*Rhododendron* sp.	MG865581	-	-
P. richardiae	**IMI 340618**	*Zantedeschia aethiopica*	MK496521	-	-
P. riparia	**CBS 132024**	Water	MG865583	JQ626607	MH136975
P. riparia	VI 3-100B9F	Water	HM004225	JQ626618	MH136975
P. rosacearum	**CBS 124696**	*Malus* sp.	EU925376	-	-
P. rosacearum	CB481	*J. communis*	*OR167222*	-	-
P. siskiyouensis	**CBS 122206**	*Lithocarpus densiflorus*	EF523386	-	-
P. syringae	**CBS 110161**	*Syringa vulgaris*	AY230190	-	-
P. syringae	**CB64**	*Salix atrocinerea*	*OR167223*	-	-
P. thermophila	**CBS 127954**	*E. marginata*	MG865593	MH494019	MH136985
P. thermophila	VHS 16164	*Banksia grandis*	EU301158	JN547614	HQ012875
P. tyrrhenica	**CBS 142301**	*Quercus* sp.	KU899188	-	-
P. versiformis	**TP13.46**	*Corymbia calophylla*	KX011279	-	-
Phytophthora sp.	**CBS 147721**	*A. incana*	OP999674	OQ067250	OQ067254
Phytophthora sp.	CB61	*A. incana*	OP999675	OQ067251	OQ067255

2.4. Phylogenetic Analysis

Molecular phylogeny based on ITS sequences was used to reconstruct evolutionary relationships among the *Phytophthora* species obtained in this study into the known clades of the genus [**17**]. Twenty ITS sequences representative of the 18 species obtained were compiled in a dataset together with 51 sequences from ex-type material of *Phytophthora* species representative of all phylogenetical clades (Table 2).

In addition, a multigene phylogeny based on concatenated ITS, Btub and *cox1* sequences of three isolates obtained in this study and other 19 formally described *Phytophthora* species in the sub-clade 6b, including ex-type cultures was performed (Table 2).

Sequences were aligned with ClustalX v. 1.83 [**46**], using the parameters reported by Bregant *et al*. [**14**].

Phylogenetic reconstructions were performed with MEGA-X 10.1.8, including all gaps in the analyses. The best model of DNA sequence evolution was determined automatically by the software [**47**]. Maximum likelihood (ML) analysis was performed with a neighbour-joining (NJ) starting tree generated by the software. A bootstrap analysis (1000 replicates) was used to estimate the robustness of nodes. Alignments and trees are available in TreeBase [studies S30526 and S30527].

2.5. Pathogenicity Test

The pathogenicity of the new *Phytophthora* species and other seven species isolated for the first time from common juniper was tested on 5-year-old *Juniperus communis* seedlings grown in plastic pots (20 cm diameter, 5 L volume). Ten 2-year branches were inoculated with each isolate, and ten were used as control. Inoculated point was surface-disinfected with 70% ethanol and a small piece of outer and inner bark (3 × 3 mm) was removed with a flamed scalpel. An agar mycelium plug of the same size (3 × 3 mm) taken from the margin of an actively growing colony (4-day-old) on PDA was placed into the wound and the inoculation point was covered with moistened cotton and wrapped in aluminium foil. Control seedlings were inoculated with a sterile PDA plug applied as described above.

All inoculated seedlings were kept in a cold greenhouse at 17 to 26 °C and watered regularly for 30 days. At the end of the experimental period, seedlings were checked for the presence of external and internal disease symptoms. The length of necrotic lesion surrounding each inoculation point was measured after removing the outer bark with a scalpel.

Re-isolation was performed by transferring 10 pieces of inner bark fragments taken around the margin of the necrotic lesions onto PDA+. Growing colonies were subcultured onto CA, incubated in the dark at 20 °C for seven days and identified by morphological and molecular analysis (ITS region).

2.6. Data Analysis

The variation in *Phytophthora* community structure among trees, shrubs and herbaceous plant species was assessed using the Jaccard similarity index (Jc) based on presence or absence of species among different microbial communities [**48**], Jc = j/(a + b + j), where j = represents the number of species in common between the two groups; a = the number of species isolated from group A; b = number of species isolated from group B.

The diversity of *Phytophthora* species associated with the three different plant types was calculated using the Margalef richness index (d) [**49**], the Shannon diversity index (H) [**50**] and the evenness index (J) [**51**]. The indices were calculated using Past software, version 4.03 [**52**].

Similarity in terms of taxonomic richness among the communities within the three plant categories was schematized through the use of Venn diagrams [**53**], using GeneVenn software to generate the diagram (https://www.bioinformatics.org/gvenn/) and reconstructing it in Canva (https://www.canva.com/).

Results of the pathogenicity test were checked for normality, then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using Fisher's least significant differences multiple range test (p = 0.05) after one-way ANOVA using XLSTAT 2008 software (Addinsoft, Paris, France).

3. Results

3.1. Symptomatology

Monitoring surveys conducted in 54 sites distributed in Italy and Slovenia allowed the occurrence of *Phytophthora*-related diseases to be detected in several plants typical of the alpine and subalpine climate. Disease incidence was highest in shrub vegetation, alpine heathlands and along the mountain riparian systems, ranging from 25 to 100%, with a mortality rate between 5 and 45% (Table 3). The most impacted ecosystems were heathlands dominated by common juniper and blueberry, and alder riparian systems (Figure 1). In these ecosystems, *Phytophthora* outbreaks showed an epidemic trend with a high mortality rate.

Many of the aerial *Phytophthora* symptoms observed were new and involved various plant organs such as leaves (moist necrotic lesions), fruit (rot), twigs (wilting and shoot blights). Moreover, on tree and shrub species stem and branches extensive bleeding cankers were observed (Figure 1). Cankers and necrosis progressively girdled the circumference of the branch, causing partial or total death of the crown.

On shrubs and heath formations, the disease was initially observed in small areas and progressively spread in a concentric manner affecting more plant species (Figure 1).

Table 3. Symptoms observed on each plant host and disease incidence/mortality rate estimated.

Plant Species	Symptoms Observed	Disease Incidence (%)	Mortality Rate (%)
Acer pseudoplatanus	Bleeding cankers, inner bark necrosis	nd *	nd
Acer monspessulanum	Bleeding cankers, inner bark necrosis	nd	nd
Alnus cordata	Foliar necrosis, shoot blight, wilting	nd	nd
Alnus glutinosa	Bleeding cankers, shoot blight, wilting	nd	nd
Alnus incana	Bleeding cankers, inner bark necrosis, shoot blight	55–100	15–35
Alnus viridis	Bleeding cankers, shoot blight, wilting, foliar necrosis, sudden death	80–100	17–42
Betula pendula	Bleeding cankers, inner bark necrosis	nd	nd
Calluna vulgaris	Shoot blight, wilting, sudden death	nd	nd
Erica carnea	Shoot blight, wilting	nd	nd
Fagus sylvatica	Bleeding cankers, inner bark necrosis	nd	nd
Fragaria vesca	Foliar necrosis, wilting	nd	nd
Fraxinus excelsior	Bleeding cankers, inner bark necrosis		
Genista corsica	Wilting, sudden death	nd	nd
Ilex aquifolium	Foliar necrosis, wilting, bleeding cankers, inner bark necrosis	100	5
Juniperus communis	Shoot blight, wilting, sudden death	25–100	3–40
Laburnum alpinum	Bleeding cankers, inner bark necrosis	80	30
Larix decidua	Shoot blight, wilting	nd	nd
Lonicera alpigena	Foliar necrosis, wilting	nd	nd
Lycopodium clavatum	Foliar necrosis, wilting, sudden death	nd	nd
Pinus mugo	Wilting, shoot blight, sudden death	20–60	5–25
Populus tremula	Foliar necrosis, shoot blight, wilting	100	-
Quercus pubescens	Bleeding cankers	100	27
Rhododendron ferrugineum	Bleeding cankers, shoot blight, wilting, foliar necrosis, sudden death	42–84	12–26
Rhododendron hirsutum	Shoot blight	nd	nd
Rubus idaeus	Foliar necrosis	nd	nd

Salix alpina	Shoot blight, wilting, sudden death	17	5
Salix atrocinerea	Bleeding cankers, inner bark necrosis	nd	nd
Salix caprea	Foliar necrosis, bleeding cankers, inner bark necrosis, epicormic shoots	68–83	14–34
Sorbus aria	Bleeding cankers, inner bark necrosis	nd	nd
Sorbus aucuparia	Bleeding cankers, inner bark necrosis, shoots blight	90	30
Taxus baccata	Shoot blight, wilting	nd	nd
Vaccinium myrtillus	Foliar necrosis, fruit rot, shoot blight. sudden death	30–80	10–45
Vaccinium vitis-idaea	Foliar necrosis, fruit rot, shoot blight, sudden death	nd	nd

* nd = Not determined.

Figure 1. Overview of aerial *Phytophthora* disease symptoms observed on: *Alnus viridis* (**1**), *Juniperus communis* (**2**), *Rhododendron ferrugineum* (**3**) *Vaccinium myrtillus* (**4**); *Alnus cordata* (**5**), *Alnus glutinosa* (**6**), *Alnus viridis* (**7**,**8**), *Juniperus communis* (**9**,**10**), *Ilex aquifolium* (**11**), *Lycopodium clavatum* (**12**), *Pinus mugo* (**13**), *Populus tremula* (**14**), *Salix caprea* (**15**), *Taxus baccata* (**16**), *Rhododendron* spp. (**17–19**), *Vaccinium* spp. (**20–22**); *Alnus* spp. (**23–25**), *Ilex aquifolium* (**26**), *Fagus sylvatica* (**27**) and *Salix caprea* (**28**). On the left, starting from the top, colony morphology of: *Phytophthora acerina*, *P. alpina*, *P. bilorbang*, *P. cactorum*, *P. cambivora*, *P. chlamydospora*, *P. gonapodyides*, *P. hedraiandra*, *P. idaei*, *P. ilicis*, *P. kelmanii*, *P. plurivora*, *P. pseudocryptogea*, *P. pseudogregata*, *P. pseudosyringae*, *P. psychrophila*, *P. rosacearum* and *P. syringae* after 7 days of growth at 20 °C on CA in the dark.

3.2. Aetiology

Isolations performed on 397 samples yielded a total of 360 *Phytophthora* isolates. Based on morphological features and ITS sequence data, 17 known *Phytophthora* species were identified, namely: *P. pseudosyringae* (201 isolates), *P. plurivora* (54), *P. gonapodyides* (21), *P. ilicis* (20), *P. alpina* (17), *P. acerina* (11), *P. cactorum* (7), *P. pseudocryptogea* (6), *P. cambivora* (5), *P. idaei* (4), *P. psychrophila* (3), *P. bilorbang* (2), *P. chlamydospora* (2), *P. hedraiandra* (1), *P. kelmanii* (1), *P. rosacearum* (1) and *P. syringae* (1).

In addition, three isolates obtained from necrotic tissues of *Alnus viridis*, *Juniperus communis* and *Rhododendron ferrugineum* could not be assigned to any known *Phytophthora* species and are therefore described here as *Phytophthora pseudogregata* sp. nov.

The assemblage and distribution of *Phytophthora* species was very variable among hosts and geographic areas. The 33 plant species monitored were divided into three main categories: small trees (Table 4), shrubs/heathland species (Table 5) and herbaceous/perennial plant species (Table 6).

The most common and widespread *Phytophthora* species detected in this study was *P. pseudosyringae.* This species was isolated from 25 out of the 33 hosts, in 36 sites distributed in all monitored geographic regions. Together with *P. cactorum*, it is the only species detected in all three types of hosts, while the other *Phytophthora* species were isolated from only one or two types (Figure 2). *Phytophthora plurivora* was the second most-isolated species, obtained from 12 hosts in 24 sites.

Table 4. Number of *Phytophthora* isolates obtained from the different plant hosts. In brackets the number of sites for each *Phytophthora* species: *Phytophthora acerina* (ACE), *P. cactorum* (CAC), *P. cambivora* (CAM), *P. gonapodyides* (GON), *P. idaei* (IDA), *P. ilicis* (ILI), *P. kelmanii* (KEL), *P. plurivora* (PLU), *P. pseudosyringae* (PSS) and *P. psychrophila* (PSY).

Tree Species	***Phytophthora* Isolates (Number of Sites)**										
	ACE	**CAC**	**CAM**	**GON**	**IDA**	**ILI**	**KEL**	**PLU**	**PSC**	**PSS**	**PSY**
Acer pseudoplatanus	-	-	-	-	-	-	-	2 (2)	-	-	-
A. monspessulanum	-	-	-	-	-	-	-	-	-	2 (1) *	-
Alnus cordata	-	-	-	-	-	-	-	-	-	1 (1) *	-
Alnus glutinosa	-	-	-	2 (2)	-	-	-	-	-	3 (2)	-
Alnus incana	-	1 (1)	1 (1) *	-	2 (1) *	-	1 (1) *	5 (3)	-	-	-
Betula pendula	-	-	-	-	-	-	-	2 (1)	-	-	-
Fagus sylvatica	-	-	-	-	-	-	-	-	-	3 (3)	-
Fraxinus excelsior	4 (3) *	-	-	-	-	-	-	5 (4)	-	1 (1) *	-
Ilex aquifolium	-	-	-	2 (1) *	-	20 (4)	-	-	-	1 (1)	-
Laburnum alpinum	-	-	2 (1) *	-	-	-	-	-	-	-	-
Larix decidua	-	1 (1)	-	-	-	-	-	-	-	1 (1) *	-
Populus tremula	-	-	-	-	-	-	-	-	2 (1) *	16 (4) *	-
Quercus pubescens	-	-	-	-	-	-	-	-	-	1 (1)	3 (1)
Salix caprea	2 (2) *	-	-	-	-	-	-	12 (6) *	-	3 (3) *	-
Sorbus aria	-	1 (1) *	-	2 (1) *	-	-	-	-	-	1 (1) *	-
Sorbus aucuparia	-	-	2 (1) *	-	-	-	-	2 (2) *	-	2 (2) *	-
Taxus baccata	-	-	-	-	-	-	-	-	-	1 (1) *	-

* New host–pathogen associations.

Table 5. Number of *Phytophthora* isolates obtained from the different plant hosts. In brackets the number of sites for each *Phytophthora* species: *Phytophthora acerina* (ACE), *P. alpina* (ALP), *P. cactorum* (CAC), *P. chlamydospora* (CHL), *P. hedraiandra* (HED), *P. plurivora*

(PLU), *P. pseudocryptogea* (PSC), *P. pseudogregata* (PSG), *P. pseudosyringae* (PSS), *P. rosacearum* (ROS) and *P. syringae* (SYR).

Shrub and Heathland Species	*Phytophthora* Isolates (Number of Sites)												
	ACE	**ALP**	**BIL**	**CAC**	**CHL**	**GON**	**HED**	**PLU**	**PSC**	**PSG**	**PSS**	**ROS**	**SYR**
Alnus viridis	-	8 (4)	-	1 (1) *	-	1 (1) *	1 (1) *	4 (2) *	2(1)	1 (1) *	22 (8)	-	-
Calluna vulgaris	-	-	-	-	-	-	-	-	-	-	3 (2) *	-	-
Erica arborea sbsp. alpina	-	-	-	-	-	-	-	-	-	-	2 (1) *	-	-
Genista corsica	-	-	-	-	-	-	-	-	-	-	1 (1) *	-	-
Juniperus communis	1 (1) *	-	2(1) *	-	-	7 (1) *	-	7 (4) *	-	1(1) *	20 (8)	1 (1) *	-
Lonicera alpigena	-	1 (1) *	-	-	-	-	-	-	-	-	-	-	-
Pinus mugo	-	-	-	-	-	-	-	3 (3) *	2(1) *	-	9 (7) *	-	-
Rhododendron ferrugineum	1 (1) *	-	-	-	-	1 (1) *	-	6 (3) *	-	1(1) *	24 (10) *	-	-
Rhododendron hirsutum	-	-	-	-	-	-	-	-	-	-	2 (1)*	-	-
Salix alpina	-	-	-	-	2 (1) *	-	-	1 (1) *	-	-	1 (1) *	-	-
Salix atrocinerea	-	-	-	-	-	-	-	-	-	-	-	-	1 (1) *
Vaccinium myrtillus	3 (2) *	7 (3) *	-	-	-	4 (2) *	-	5 (3) *	-	-	67 (14)	-	-
Vaccinium vitis-idaea	-	1 (1) *	-	-	-	2 (1) *	-	-	-	-	2 (2)	-	-

* New host–pathogen associations.

Table 6. Number of *Phytophthora* isolates obtained from the herbaceous plant hosts. In brackets the number of sites for each *Phytophthora* species: *Phytophthora cactorum* (CAC), *P. idaei* (IDA) and *P. pseudosyringae* (PSS).

Herbaceous and Perennial Species	*Phytophthora* Isolates (Number of Sites)		
	CAC	**IDA**	**PSS**
Fragaria vesca	3 (2)	-	-
Lycopodium clavatum	-	-	12 (1) *
Rubus idaeus	-	2 (1)	-

* New host–pathogen associations.

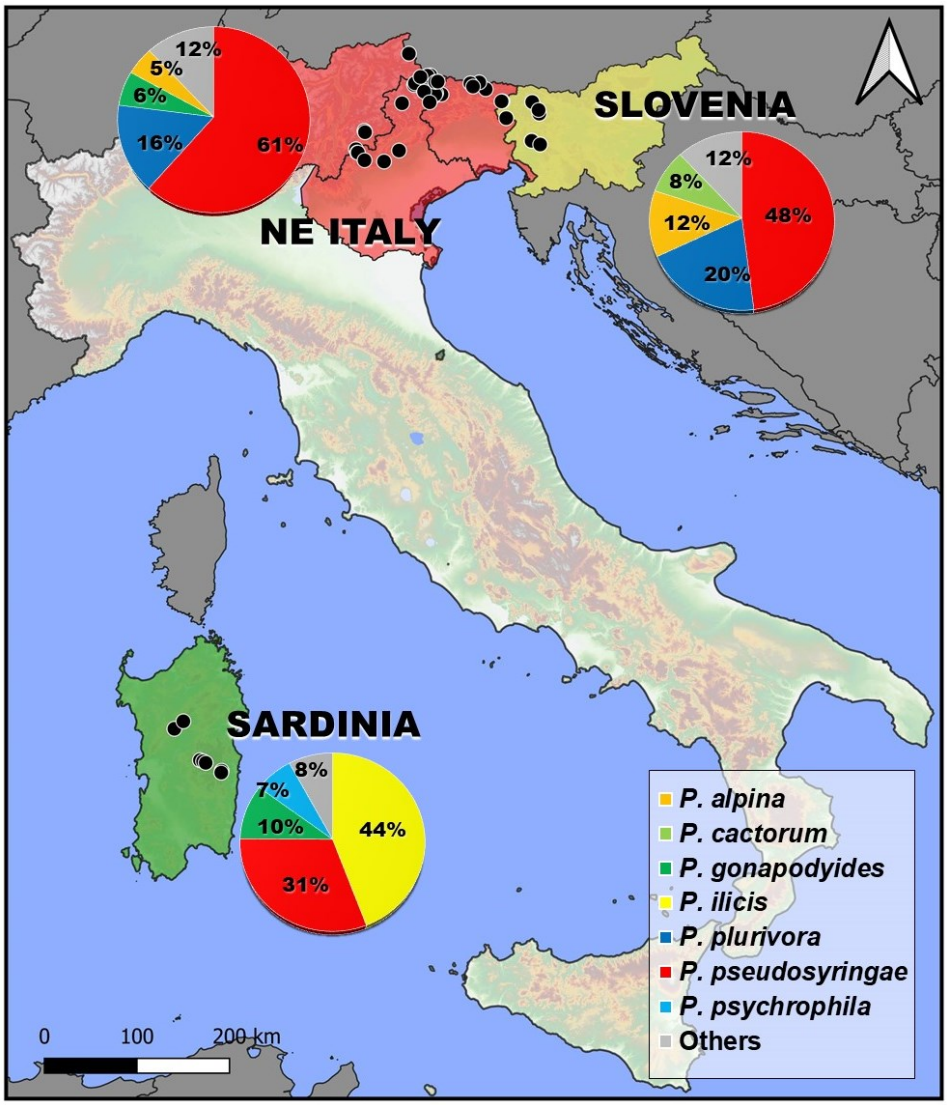


Figure 2. Isolation frequency and distribution of the 7 most common *Phytophthora* species isolated in this study.

Phytophthora pseudosyringae and *P. plurivora* were the most frequently isolated species in NE Italy and Slovenia (Figure 2). In addition to these two species, some species belonging to clade 1, such as *P. alpina* and *P. cactorum*, were frequently isolated from different hosts in the NE Alps. In the mountainous areas of Sardinia, in addition to *P. pseudosyringae*, other two species *P. ilicis* and *P. psychrophila* belonging to clade 3 were constantly isolated (Figure 2).

As regards the distribution within *Phytophthora* clades, clade 6 is the most represented in terms of species (five species) followed by clade 1 (4), clade 3 (3) and clade 8 (3). Only one or two species were obtained for clades 2 and 7. Overall, 56 new host–pathogen associations were detected (Tables 4–6).

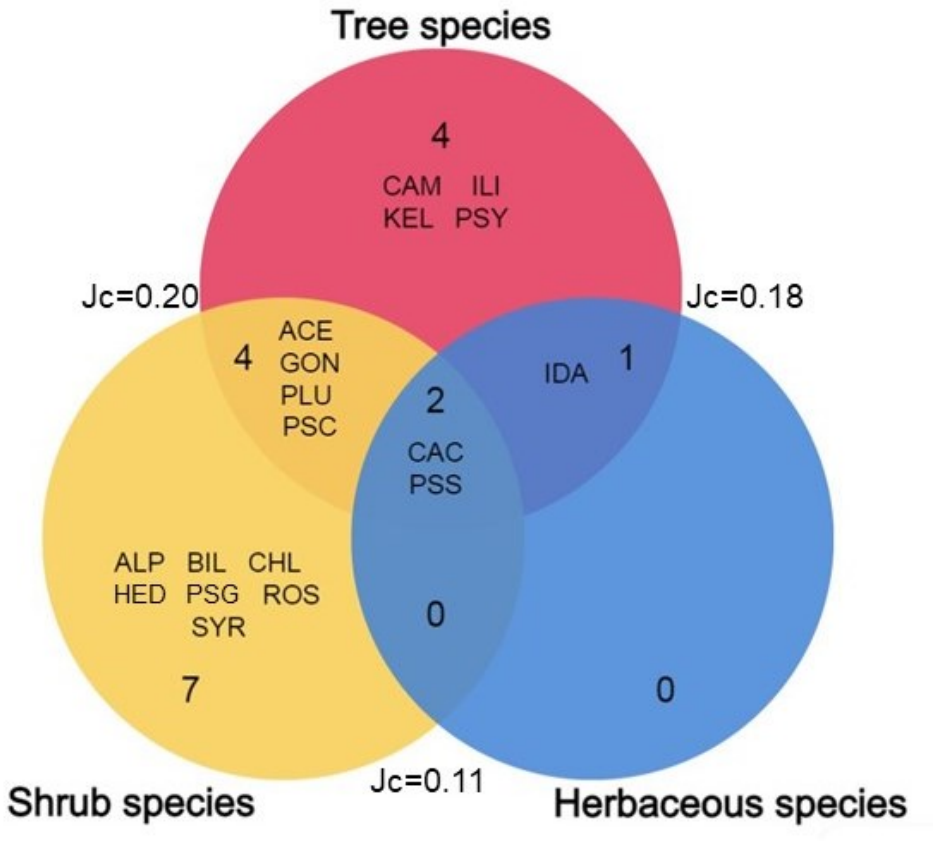
3.3. Structure and Diversity of Phytophthora Communities

The diversity indices of the *Phytophthora* assemblages detected in the subalpine vegetation varied among the three categories of hosts, but in general they displayed high diversity and richness and moderate evenness, with the exception of the shrub *Phytophthora* community dominated by *P. pseudosyringae* (Table 7).

Tree and shrub species displayed the highest number of taxa and Shannon index (H) values. As regards the degree of similarity between the three *Phytophthora* communities, the Jaccard similarity index (Jc) was variable between 0.11 and 0.20. Only two *Phytophthora* species, *P. pseudosyringae* and *P. cactorum*, were isolated from all host groups. Relationships among the three categories of hosts are shown in Figure 3.

Table 7. Values of the diversity indices, Shannon diversity index (S), Margalef index (d) and Pielou Evenness (J) of *Phytophthora* populations from three different plant communities.

Plant Types	Taxa	Shannon Index (H)	Margalef (d)	Pielou Evenness (J)
Tree species	11	1.867	2.158	0.588
Shrub species	13	1.348	2.227	0.296
Herbaceous species	3	0.804	0.706	0.745



ACE= *P. acerina*; **ALP**= *P. alpina*; **BIL**= *P. bilorbang*; **CAC**= *P. cactorum*; **CAM**= *P. cambivora*; **CHL**= *P. chlamydospora*; **GON**= *P. gonapodyides*; **HED**= *P. hedraiandra*; **IDA**= *P. idaei*; **ILI**= *P. ilicis*; **KEL**= *P. kelmanii*; **PLU**= *P. plurivora*; **PSC**= *P. pseudocryptogea*; **PSG**= *P. pseudogregata*; **PSS**= *P. pseudosyringae*; **PSY**= *P. psychrophila*; **ROS**= *P. rosacearum*; **SYR**= *P. syringae*.

Figure 3. Venn diagrams illustrating the number of unique and shared *Phytophthora* species among the three categories of plant species. The outer numbers indicate values of the Jaccard similarity coefficient.

3.4. DNA Phylogeny

Phylogenetic relationships among the *Phytophthora* isolates obtained in this study were elucidated using ITS sequences (Figure 4). In particular, the 20 isolates included in the phylogenetic analysis were distributed in 18 terminal clades, 17 of which belong to formally described species (Figure 4). Instead, three isolates clustered together in a separate and well-supported terminal clade (ML

bootstrap = 100%) representing a previously unrecognized species closely related to *P. gregata*, which is described here as *Phytophthora pseudogregata* sp. nov. (Figure 4).

To resolve the phylogenetic position of *P. pseudogregata* within subclade 6b, a concatenated nuclear and mitochondrial dataset (the length of the final alignment was 2129 bp) was analysed. Individual gene phylogenies revealed no major conflicts, thus indicating that the three loci (ITS, Btub and *cox*1) could be combined. The ML analysis resolved the positions of all formally described *Phytophthora* species in subclade 6b, accommodating the isolates *P. pseudogregata* in a terminal clade sister to *P. gibbosa* (Figure 5). *Phytophthora pseudogregata* is separated by the two closely related species, *P. gregata* and *P. gibbose*, by three, two, and 18 bp and by eight, three, and 17 bp in ITS, Btub, and *cox*1 loci, respectively.

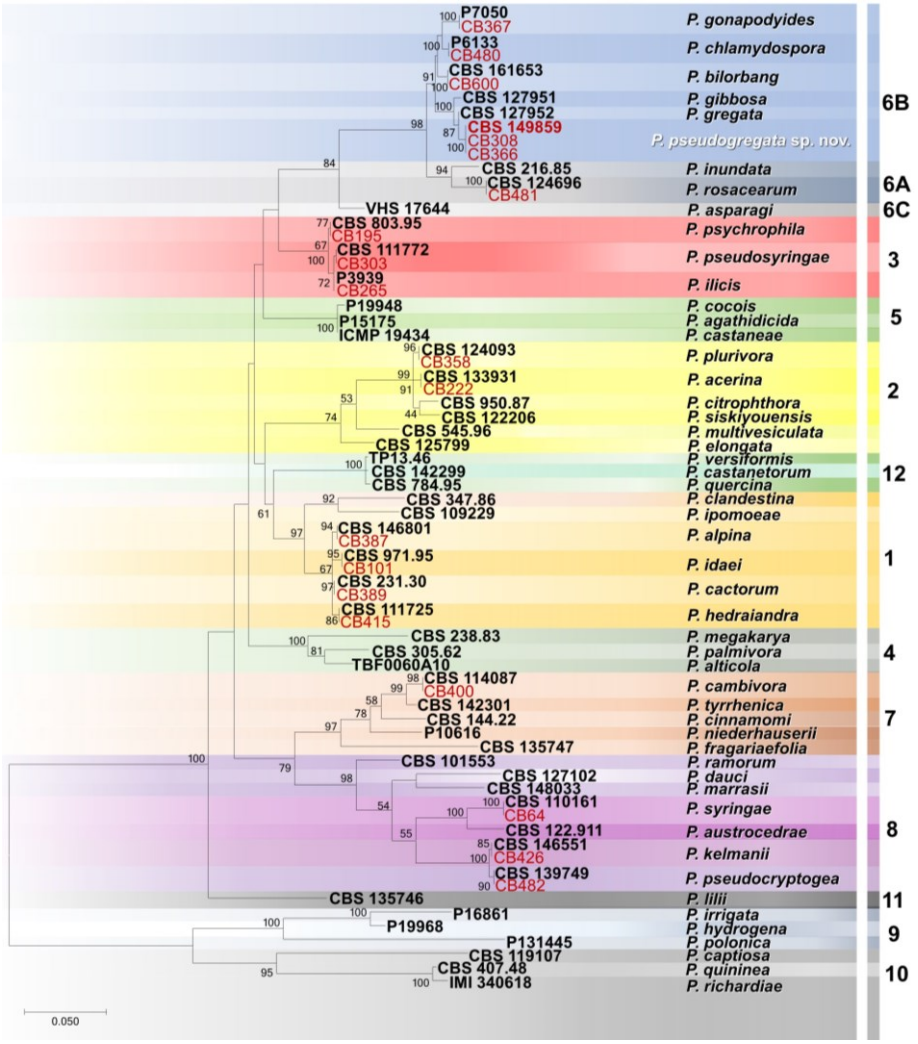


Figure 4. Maximum likelihood tree obtained from internal transcribed spacer (ITS) sequences of *Phytophthora* species representative of the 12 clades. Data are based on the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap support values in percentage (1000 replicates) are given at the nodes. Ex-type cultures are in bold, and isolates obtained in this study in red.

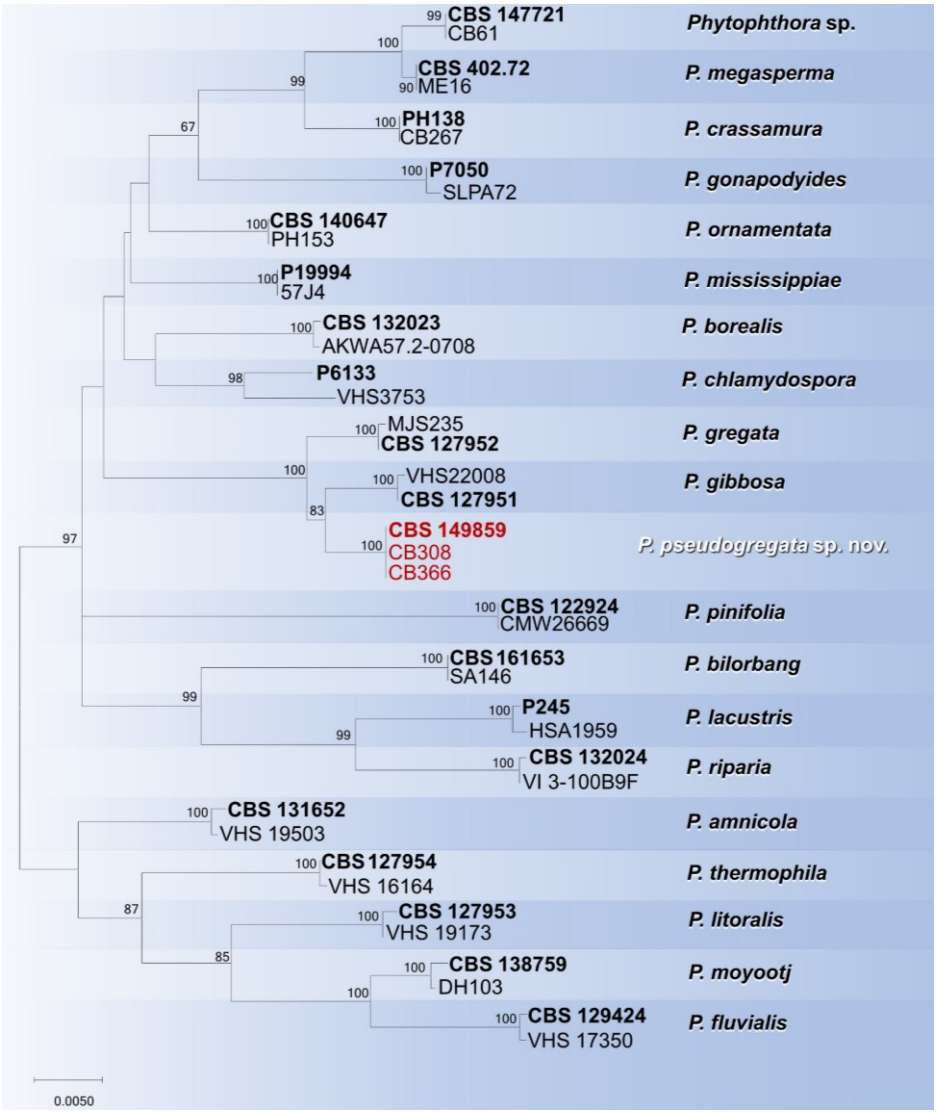


Figure 5. Maximum likelihood tree obtained from concatenated ITS, Btub and *cox*1 sequences of the *Phytophthora* species belonging to subclade 6b. Data are based on the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap support values in percentage (1000 replicates) are given at the nodes. Ex-type cultures are in bold and isolates of the study in red.

3.5. Taxonomy

Phytophthora pseudogregata Bregant, Ogris, Meli and Linaldeddu sp. nov.

MycoBank: MB849354

Etymology: the name refers to the morphological similarity to *Phytophthora gregata*.

Holotype: CBS H-25226

Host/distribution: *Alnus viridis*, *Juniperus communis* and *Rhododendron ferrugineum* with foliar necrosis and shoot blight symptoms in Italy and Slovenia.

Description: Sporangia were produced on CA plugs flooded in unsterile pond water after 36–72 h of incubation at 25 °C on simple sporangiophores. Sporangia were persistent, mostly nonpapillate (80%), rarely semipapillate (20%), from ovoid to obpyriform, sometimes ellipsoid, borne terminally on unbranched sporangiophores, average 50.3 ± 6.5 × 29.9 ± 3.8 µm (total range 32.1–

65.1 × 22.1–38.4 µm), with a length/breadth ratio of 1.7 ± 0.2 (*n* = 50) (Figure 6d–g). Zoospores were abundantly produced in liquid cultures after 24–36 h at 25 °C in the dark (Figure 6h). Sporangia proliferated, usually externally and rarely internally, in both a nested and extended way (Figure 6i–k). Hyphal swellings were not formed on solid agar and rarely in pond water, they were globose to subglobose, mostly intercalary catenulate, rarely terminal (Figure 6l–m). Chlamydospores were not observed. All isolates produced gametangia in single culture on carrot agar after 7–10 days at 20 °C in the dark. Oogonia were smooth-walled, borne mainly terminally, with an average diameter of 33.2 ± 3.4. Oospores were spherical and usually aplerotic and 29.0 ± 3.7 µm in diameter. Antheridia were mostly amphigynous (58%), less frequently paragynous (42%) hyaline, rounded, club-shaped, or irregular: average 16.2 ± 2.7 × 12.5 ± 2.2 µm (Figure 6o–r).

Cultural characteristics: colony growth pattern cottony on PDA with an irregular border, with an indistinct pattern on MEA and CA. On PDA, growth was slow, whereas on MEA and CA, colonies reached a diameter of 55 and 70 mm in 7 days at 23 °C, respectively.

Cardinal temperatures for growth: minimum <2 °C, maximum 32 °C, and optimum 23 °C. Isolates failed to grow at 34 °C, and mycelium did not resume growth when plates were moved to 20 °C.

Material examined: ITALY: Borso del Grappa, isolated from a necrotic shoot of *Juniperus communis*, 13 June 2022, collected by Letizia Meli, isolated by Carlo Bregant, HOLOTYPE CBS H-25226, a dried culture on CA, culture ex-holotype CB234 = CBS 149859. ITALY: San Nicolò di Comelico, isolated from necrotic leaves of *Rhododendron ferrugineum*, 3 July 2021, collected and isolated by C. Bregant (isolate CB308). SLOVENIA: Bohinj, isolated from a necrotic branch of *Alnus viridis*, 7 October 2021, collected by C. Bregant and Nikica Ogris and isolated by C. Bregant (isolate CB366).

Notes: *Phytophthora pseudogregata* belongs to subclade 6b. The closest species are *P. gregata* and *P. gibbosa*, from which it differs through a combination of unique morphological features (Table 8) and sequence data such as sporangia size and proliferation, oogonia and antheridia shapes and cardinal temperature values, as well as a total of 23 (*P. gregata*) and 28 (*P. gibbosa*) fixed nucleotide differences in the ITS, Btub, and *cox1* sequences.

Figure 6. Colony morphology of *Phytophthora pseudogregata* after 7 days growth at 20 °C on PDA (**a**), MEA (**b**), and CA (**c**). Persistent sporangia, nonpapillate (**d**,**e**) semipapillate (**f**,**g**), releasing of zoospores (**h**), external (**i**) and internal (**j**,**k**) proliferations; intercalary (**l**) and terminal hyphal swellings (**m**); mycelia (**n**). Oogonia with amphygynous (**o**,**p**) and paragynous antheridia (**q**,**r**). Scale bars = 20 μm.

Table 8. Morphological features, morphometric data and temperature–growth relationship of *Phytophthora pseudogregata* and closely related species in subclade 6b.

	P. pseudogregata	***P. gregata***	***P. gibbosa***
Number of isolates examinated	3	**[54]**	**[54]**
Sporangia	Ovoid to obpyriform, sometimes ellipsoid, nonpapillate, some semipapillate	Ovoid, limoniform, obpyriform, nonpapillate	Ovoid, ellipsoid, nonpapillate, some semipapillate
Length × breadth mean (μm)	50.3 ± 6.5 × 29.9 ± 3.8	51.0 ± 13.8 × 30.5 ± 5.9	48.8 ± 9.6 × 30.8 ± 5.4
Total range	32.1–65.1 × 22.1–38.4	25.7–102.3 × 14.8–50.7	24.8–71.1 × 17.4–48.0
Length/Breadth ratio	1.7 ± 0.2	1.67 ± 0.32	1.58 ± 0.15

Proliferation	Mostly external, sometimes internal, mostly extended and rarely nested	Internal extended and nested, never external, sporangiophore partly branching inside empty sporangium	Internal extended, external, never nested
Hyphal swellings	Globose to subglobose, mostly intercalary catenulate, rarely terminal	Globose, elongated, angular, partly catenulate	Subglobose, elongated, never catenulate
Chlamydospores	Not observed	Not observed	Not observed
Breeding system	Homothallic	Homothallic or self-fertile	Homothallic
Oogonia	Smooth	Smooth	Ornamented, smooth
Mean diameter (μm)	33.2 ± 3.4	36.8 ± 4.1	38.1 ± 5.4
Diameter range (μm)	26.9–41.6	23.9–50.9	27.0–49.9
Oospores	Aplerotic	Usually aplerotic	Always aplerotic
Mean diameter (μm)	29.1 ± 3.7	31.6 ± 4.0	31.4 ± 4.6
Total range (μm)	20.5–37.8	21.4–45.3	18.9–39.4
Wall thickness (μm)	2.18 ± 0.71	2.65 ± 0.81	3.17 ± 0.69
Antheridia	Mostly amphygynous (58%), less frequently paragynous (42%)	Mostly paragynous	Amphigynous
Length × breadth mean (μm)	16.2 ± 2.7 × 12.5 ± 2.2	17.1 ± 3.0 × 11.0 ± 1.8	13.6 ± 2.4 × 14.0 ± 2.0
Total range (μm)	11.7–23.3 × 9.0–17.9	10.6–24.9 × 7.6–17.8	10.6–24.9 × 7.6–17.8
Maximum temperature (°C)	32	32.5≤35	32.5≤35
Optimum temperature (°C)	23	25	30

3.6. Pathogenicity

All *Phytophthora* species proved to be pathogenic on *Juniperus communis*. At the end of the experimental period, inoculated seedlings showed dark brown inner bark lesions that spread up and down from the inoculation point (Figure 7).

Among the different species assayed, the length of the necrotic lesion differed significantly (Table 9). The lesions caused by *P. pseudosyringae* were significantly larger than those caused by other species (Table 9). Lesions caused by *P. pseudosyringae*, *P. plurivora* and *P. acerina* progressively girdled the twigs causing shoot blight, browned foliage and wilting symptoms.

Control seedlings, inoculated with sterile PDA plugs, remained symptomless; in only two twigs, a small light brown discoloration was observed restricted to the inoculation point.

All eight *Phytophthora* species were successfully re-isolated from the necrotic inner bark lesions of all seedlings, thus fulfilling Koch's postulates. No *Phytophthora* or other fungal isolates were obtained from control plants.

Table 9. Mean lesion length ± standard deviation caused by each *Phytophthora* species on common juniper twigs.

Species	Isolate	Mean Lesion Length (cm)*	Wilted Foliage	Re-Isolation (%)
P. acerina	CB222	1.4 ± 0.2 e	yes	100
P. bilorbang	CB600	1.1 ± 0.7 f	no	80
P. gonapodyides	CB367	1.4 ± 0.3 de	no	100
P. plurivora	CB358	1.9 ± 0.2 b	yes	100

P. pseudocryptogea	CB482	1.7 ± 0.3 c	no	100
P. pseudogregata	CBS149859	1.7 ± 0.2 cd	no	100
P. pseudosyringae	CB303	2.3 ± 0.4 a	yes	100
P. rosacearum	CB481	1.3 ± 0.2 ef	no	100
Control	-	0.2 ± 0.1 g	no	-
LSD critical value		1.90		

* Values with the same letter do not differ significantly at $p = 0.05$, according to LSD multiple range test.

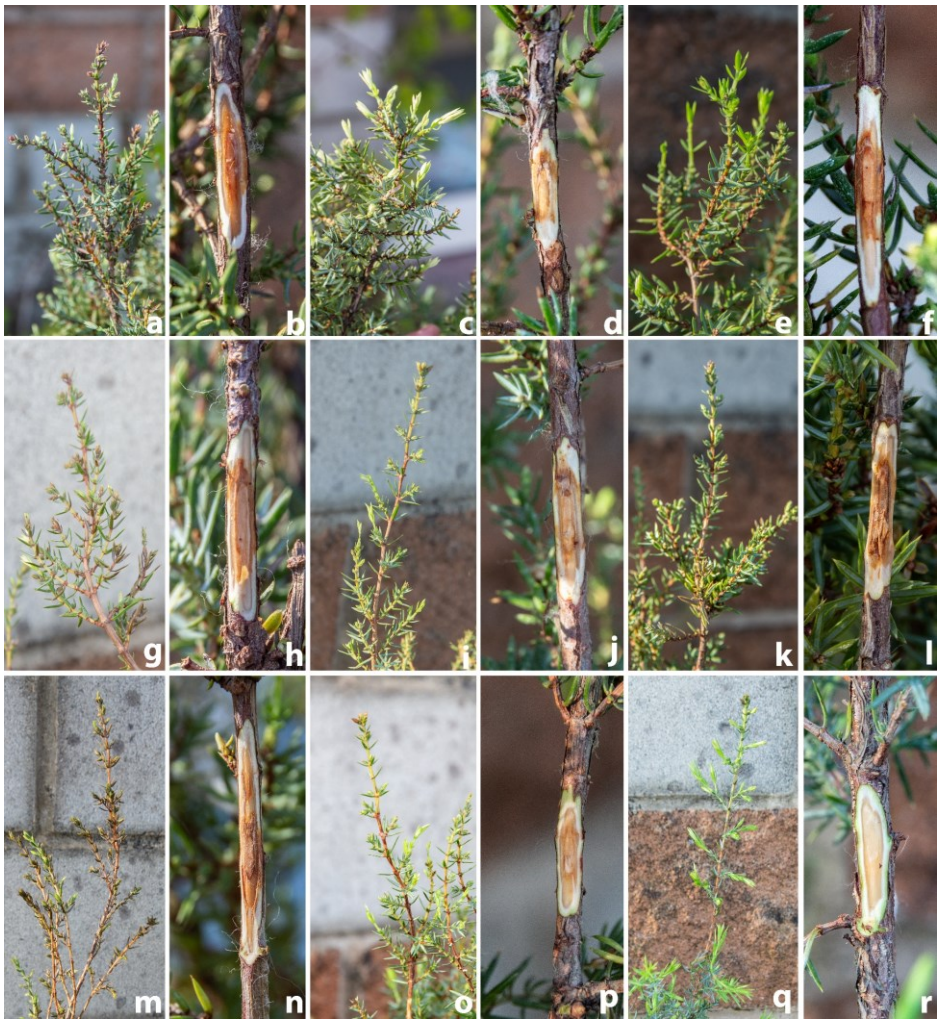


Figure 7. Symptoms observed on common juniper twigs 30 days after inoculation with *Phytophthora acerina* (**a**,**b**), *P. bilorbang* (**c**,**d**), *P. gonapodyides* (**e**,**f**), *P. plurivora* (**g**,**h**), *P. pseudocryptogea* (**i**,**j**), *P. pseudogregata* (**k**,**l**), *P. pseudosyringae* (**m**,**n**) and *P. rosacearum* (**o**,**p**). Control (**q**,**r**).

4. Discussion

This study represents the most comprehensive investigation to date on aerial diseases caused by *Phytophthora* species on mountain vegetation in Italy and Slovenia. The results obtained have allowed us to clarify both symptomatology and aetiology of the emerging pathosystems affecting mountain and subalpine formations. The progressive spread of several airborne *Phytophthora* species is causing the destruction of vast ecosystems and compromising the biodiversity of these ecologically fragile habitats.

Based on combined sequence data and micromorphological features, 18 *Phytophthora* species belonging to six out the 12 major *Phytophthora* phylogenetic

clades were identified from a collection of 397 symptomatic samples collected from 33 herbaceous and woody hosts. These included: *P. acerina*, *P. alpina*, *P. bilorbang*, *P. cactorum*, *P. cambivora*, *P. chlamydospora*, *P. gonapodyides*, *P. hedraiandra*, *P. idaei*, *P. ilicis*, *P. plurivora*, *P. pseudocryptogea*, *P. pseudosyringae*, *P. psychrophila*, *P. rosacearum* and *P. syringae*. In addition, three isolates described here as *Phytophthora pseudogregata* sp. nov. were isolated and characterized.

The most frequently isolated *Phytophthora* species belong mainly to clades 1 and 3. These species are characterized by the ability to produce caducous sporangia useful for aerial infections [**1**]. Furthermore, the relatively low cardinal temperatures for growth suggest that these species have a great potential to threaten mountain vegetation [**14**,**31**,**55**].

In particular, in Northeast Italy a higher number of species belonging to the ITS clade 1 was isolated (*P. alpina*, *P. cactorum*, *P. hedraiandra* and *P. idaei*), while in Sardinia, clade 3 was dominant, with three species, *P. pseudosyringae*, *P. ilicis* and *P. psychrophila*. Overall, *P. pseudosyringae* (clade 3) was the most frequent species in terms of number of hosts infected and distribution among sites. Two hundred and one out of the 360 isolates obtained in this study belonged to this species. In particular, *P. pseudosyringae* have been detected in 36 sites and 25 hosts of all three plant categories investigated: trees, shrubs and herbaceous plants (17 new host–pathogen associations). The wide spread of *P. pseudosyringae* in different mountain and subalpine formations and its involvement in several new diseases highlight the polyphagous nature of this invasive pathogen and its aerial lifestyle. This agrees with previous studies conducted in mountain environments in Asia, Europe, North and South America [**14**,**32**,**34**,**55–61**]. *Phytophthora pseudosyringae* is the key species in the aetiology of aerial infections detected in high-altitude shrubs and heaths such as blueberry, dwarf pine, juniper, rhododendron and alpine willow formations; these shrubs are characterized by creeping behaviour with very limited heights above the ground, this habitus could favour the attack of *Phytophthora* sporangia and zoospores. The attacks of *P. pseudosyringae* on *Vaccinium myrtillus* (leaf necrosis and shoot blight) were particularly severe, confirming the susceptibility of this small shrub as previously reported in Ireland [**33**,**62**]. Many aspects regarding the infectivity and survival of *P. pseudosyringae* sporangia in the infected tissues fallen to the ground in subalpine areas remain to be clarified. At the same time the ability of oospores to persist for years and their infectivity in environments where the pathogen is subjected to extreme low temperatures need further investigations. Probably the survival of this species in cold habitats is guaranteed by the production of very large and thick wall chlamydospores. In fact, unlike what was reported in previous studies and in the original description, all isolates of *P. pseudosyringae* examined produced a large amount of globose chlamydospores on CA both in solid and liquid culture. The chlamydospores were mainly terminal and 76.6 ± 22.02 (range 39.9–102.3, n = 25) μm in diameter. Based on the wide variation in morphological characters found in this study, the description of *P. pseudosyringae* needs to be redefined. Undoubtedly, increased inoculum in the litter due to the diseased fallen leaves not only could represent an increased risk of outbreaks but also a faster disease progression in these habitats [Bregant & Linaldeddu, unpublished]. In the pathogenicity test, *P. pseudosyringae* shows high aggressivity on common juniper, producing wood necrosis and shoot blight after four weeks from the inoculation.

The other two species in clade 3 were isolated only in the mountain area of Sardinia. *Phytophthora psychrophila* have been isolated from bleeding cankers of *Quercus pubescens,* confirming the affinity of this pathogen towards oak species [**63**]; the geographic distribution and impact of this species is still unknown; there have been a few reports of it in European and American natural forests and

nurseries [**4**,**31**,**64**]. *Phytophthora ilicis* has been known for a long time as a specific pathogen of *Ilex aquifolium* in the mountains of the Mediterranean basin and a few other areas of Europe and North America [**55**,**65–67**].

Four species belonging to subclade 1a have been isolated in the northeastern Alps. *Phytopththora alpina* shows the highest ability to survive in extremely cold conditions due to the low temperature values for growth and the high production of caducous sporangia and chlamydospores [**14**]; in addition to *Alnus viridis*, its discovery on three new hosts in Italy and Slovenia suggest that this recently described species is well adapted to affect typical alpine and subalpine shrubs. The second most common species in subclade 1a was *P. cactorum*, an invasive and polyphagous pathogen widespread from tropical to temperate climates where it is responsible for severe diseases on agriculture crops and forest trees [**1**,**29**,**68**]. The occurrence of *P. cactorum* in cold areas has recently been reported in Europe and Australia [**4**,**14**,**60**,**69**]. Together, *P. pseudosyringae* and *P. cactorum* are the two species obtained from all three plant types.

In addition to the numerous new host–pathogen associations (Table 4–6), some species detected such as *P. hedraiandra* and *P. idaei* are reported for the first time in natural ecosystems in Europe. Previous studies have ascertained the involvement of these two pathogens in root and foliar disease in agriculture and ornamental nurseries; *P. idaei* appears restricted to the genus *Rubus* [**70**], while *P. hedraiandra* has a wider range of ornamental hosts [**71–75**]. Although, in the original description *P. idaei* is reported to have persistent-sporangia, the Italian isolates obtained in this study showed a moderate production of caducous sporangia.

The second most common species obtained in this study was *P. plurivora*. Isolates of this species were obtained from 54 symptomatic samples of 12 plant species including eight new hosts. *Phytophthora plurivora* resides in clade 2 and is common in forest ecosystems of Central Europe; from a recent population study it is considered to be originally of this continent and spread to others by human activities [**76**]. This agrees with the results of this and previous studies [**8**,**14**] given the wide distribution of this pathogen in various extreme and nonhumanized natural environments. While the distribution and impact of *P. plurivora* is well studied, little is known about its closely related species, *P. acerina*. To date, this species appears widespread in agricultural systems, nurseries, forests and ornamental trees in northern Italy and Sardinia, and much rarer worldwide [**4**,**77–79**]. Both *P. acerina* and *P. plurivora* were already known as primary pathogens involved in common and grey alder decline in Italy [**14**]. Isolates of *Phytophthora acerina* obtained in this study confirm a single polymorphism in the ITS region between northern Italy and Sardinia populations [**14**].

Among the other *Phytophthora* species isolated in this study, five belong to clade 6, including the newly described species *P. pseudogregata*. Clade 6 encompasses species very common in European forests, such as *P. bilorbang* and *P. gonapodyides* and species with more limited or still unknown distribution, such as *P. amnicola* and *P. rosacearum* [**8**]. Some species in this clade are reported as saprophyte or occasionally weak opportunistic pathogens [**11**,**54**,**80**,**81**]; the involvement of five species of this clade in the aetiology of aerial infections on mountain vegetation highlight the ecological versatility of these organisms. The ability of *P. bilorbang*, *P. gonapodyides* and *P. pseudogregata* to reproduce the symptoms observed in nature on common juniper suggest their active role in the aetiology of the emerging disease affecting woody trees in mountain areas.

Phytophthora pseudogregata resides in subclade 6b; it is closely related to *P. gregata* and *P. gibbosa*, from which it can be distinguished by unique

morphological features and sequence data. *Phytophthora gregata* was originally described in 2011 in Australia in wet native forests and in Tasmania associated with dying alpine heathland vegetation [**54**,**69**] and then recently reported in the Czech Republic and Finland [**60**,**82**], whereas *P. gibbosa* is known to occur only in Australia associated with dying native vegetation on seasonally wet sites [**54**].

Sub-clade 6b is larger and contains several described species (*P. amnicola*, *P. borealis*, *P. chlamydospora*, *P. bilorbang*, *P. crassamura*, *P. fluvialis*, *P. gibbosa*, *P. gonapodyides*, *P. gregata*, *P. lacustris*, *P. litoralis*, *P. megasperma*, *P. mississippiae*, *P. moyootj*, *P. ornamentata*, *P. pinifolia*, *P. pseudogregata*, *P. riparia* and *P. thermophila*), some not formally described species and a few hybrids [**83**]. Most of the species in this sub-clade have been described in the last 12 years, the only species known until 2011 were *P. gonapodyides* and *P. megasperma* [**1**]. The majority of species in sub-clade 6b, including *P. pseudogregata*, have been described in forest ecosystems, underlining the key role played by natural areas in exploring the biodiversity of the *Phytophthora* genus, which currently includes 220 species (Figure 8).

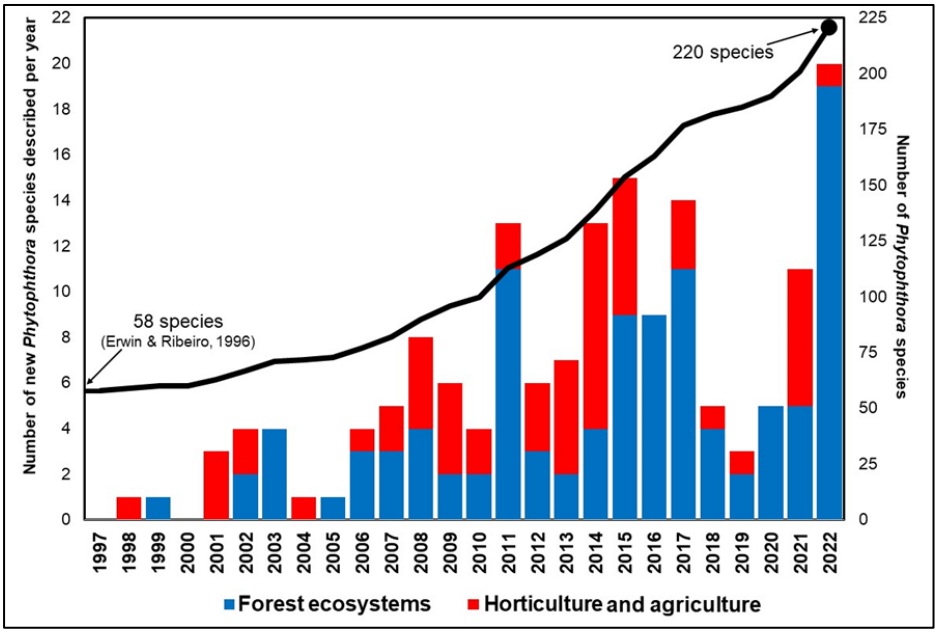


Figure 8. Number of *Phytophthora* species described per year since 1997, divided into species isolated from nurseries and agriculture and forest ecosystems. The graph also reports the progression of the described species over time (black line). (Source: Scopus, May 2023, IndexFungorum May 2023 and [**1**]).

Finally, three species of clade 8 (*P. kelmanii*, *P. pseudocryptogea* and *P. syringae*) and one from clade 7 (*P. cambivora*) have been isolated, mainly from stem bleeding cankers of small trees and shrubs. While *P. kelmanii* and *P. syringae* have a very limited distribution, *P. pseudocryptogea* is widespread along the Alps. The large range of growth temperatures and polyphagous nature explain it being widespread in Italian ecosystems spanning from Mediterranean areas to the tree line in the Dolomites [**4**,**14**,**78**]. Both mating types of *P. cambivora* occurred in the NE Alps (A2 on *Alnus incana* in Slovenia and A1 on *Laburnum alpinum* and *Sorbus aucuparia* in Italy).

5. Conclusions

In conclusion, the discovery of several emerging *Phytophthora* diseases in the canopy of subalpine vegetation in Europe is of particular concern and

underlines the need to further extend research into these environments to assess the full diversity of *Phytophthora* clades and species and the factors driving the emergence and diffusion of these invasive pathogens. Studying *Phytophthora* communities on necrotic leaves naturally fallen, would be useful to evaluate host specificity, geographic distribution and survival strategies of the main *Phytophthora* species detected in this study.

A survey is currently in progress to map the distribution of *P. pseudogregata* in Alpine habitats and to establish the natural host range of this new *taxon*.

Author Contributions: B.T.L., C.B. and N.O. conceptualization; B.T.L., C.B., G.R., L.M. (Letizia Meli), N.O., N.S. and A.B. field survey, sample collection and assay; B.T.L., C.B., G.R. and L.M. (Letizia Meli) data analysis; B.T.L., L.M. (Lucio Montecchio), L.M. (Lucia Maddau), B.P. and N.O. funding acquisition; C.B. draft writing; B.T.L., L.M. (Lucio Montecchio), L.M. (Lucia Maddau), A.B., B.P. and N.O. review and manuscript editing. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: This research was partially funded by grant number DOR2305524/2023 "Monitoraggio dei marciumi radicali da *Phytophthora* negli ecosistemi forestali Italiani", by the Land Environment Resources and Health (L.E.R.H.) doctoral course (University of Padova), by Fondo di Ateneo 2020, an internal funding by the University of Sassari and by Slovenian Research Agency (Research Program P4-0107 Forest Biology, Ecology and Technology), Slovenian Ministry of Agriculture, Forestry and Food (Public Forestry Service).

Conflicts of Interest: The authors declare no conflicts of interest.

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Chapter II

Diversity and distribution of *Phytophthora* species across different types of riparian vegetation in Italy with the description of *Phytophthora heteromorpha* sp. nov.

Bregant C., Rossetto G., Sasso N., Montecchio L., Maddau L., Linaldeddu B.T. 2023. Diversity and distribution of *Phytophthora* species across different types of riparian vegetation in Italy with the description of *Phytophthora heteromorpha* sp. nov. (submitted).

Diversity and distribution of *Phytophthora* species across different types of riparian vegetation in Italy with the description of *Phytophthora heteromorpha* sp. nov.

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Abstract

Riparian formations encompass a diverse suite of transitional zones between terrestrial and aquatic ecosystems. During the last decades, these formations have been impacted by several emerging diseases. The first outbreaks were detected on alder formations but have progressively also been observed on other plant species such as *Betula pubescens*, *Nerium oleander*, *Populus alba*, *Salix alpina*, *S. purpurea* and *Tamarix gallica*. Declining plants showed a plethora of symptoms (leaf spot, shoot blight, bleeding cankers and root rot) indicative of *Phytophthora* infections. Since there is little information about the aetiology of these pathosystems, from November 2019 to March 2023, an in-depth study was conducted in forty-six riparian ecosystems spanning from the Mediterranean to Alpine regions. Overall, 744 symptomatic samples (stem bleeding cankers and root with rhizosphere) from 27 host species were collected for *Phytophthora* isolation. Based on morphology and DNA sequence data, 20 known *Phytophthora* species belonging to seven phylogenetic clades have been identified: *P. plurivora* (202 isolates), *P. gonapodyides* (156), *P. pseudosyringae* (84), *P. lacustris* (57), *P. acerina* (31), *P. idaei* (30), *P. alpina* (20), *P. pseudocryptogea* (19), *P. cambivora* (13), *P. pseudotsugae* (13), *P. cactorum* (9), *P. hydropathica* (6), *P. debattistii* (4), *P. multivora* (4), *P. cinnamomi* (3), *P. bilorbang* (2) *P. crassamura* (2), *P. ilicis* (2) and *P. inundata* (2),. In addition, 26 isolates of a new putative species obtained from *Alnus incana* and *Pinus sylvestris* are described here as *Phytophthora heteromorpha* sp. nov. The new species proved to be pathogenic on grey alder causing symptoms congruent with field observations. This study represents the most comprehensive investigation on the *Phytophthora* species associated with declining riparian vegetation in Italy and highlights that the polyphagous pathogen *P. plurivora* represents a growing threat to Mediterranean, temperate and alpine ecosystems.

Keywords: emerging disease – oomycetes – pathogenicity – biodiversity

Introduction

Riparian habitats are transition areas between terrestrial and aquatic ecosystems and are characterized by a complex system of habitats closely related to the surrounding land environments (Naiman *et al*., 2005). They are strongly dynamic ecotones with a high spatial and temporal variability driven by natural and human influences, such as the geomorphology, climatic conditions and degree of land use (Dufour *et al*., 2019). Riparian formations provide a wide range of ecosystem services, which directly or indirectly benefit humans, including the provision of biomass and food and protection from landslides and floods (Riis *et al*., 2020). Some of these functions have proven to be fundamental for moderating the local effects of global changes, mainly the thermal conditions of watercourses and mitigation of extreme rainfall events (Kristensen *et al*., 2015; Trimmel *et al*., 2018). Riparian formations are composed of a huge number of habitats often representing floristic and faunistic corridors protected by legislation (Rosemberg *et al*., 1997; de la Fuente *et al*., 2018; Urbanič *et al*., 2022).

The assortment of plants typically consists of emergent aquatic species, or herbs, trees and shrubs that thrive in proximity to water. Plant composition varies in relation to the climate, soil and human influence (Corbacho *et al*., 2003; Fierro *et al*., 2017). In the temperate zone of the Northern hemisphere, pioneer riparian woodlands are mainly dominated by trees and shrubs belonging to the *Betulaceae* and *Salicaceae* families (Politti *et al*., 2018; Ren *et al*., 2010). In Europe, the genera *Alnus, Betula, Populus* and *Salix* are in the majority of cases the most common species along rivers and streams, starting from the Mediterranean areas to the high altitude and latitude cold environments; these plant groups are composed of a wide range of traits suitable to resist the high dynamisms of a riparian environment, including flood tolerance, an ability to survive and regenerate following damage by floods and to colonize exposed bars through clonal reproduction and fast growth rates (Lytle & Poff, 2004; Politti *et al*., 2018).

Despite the extreme adaptability to rapid environmental modifications, riparian systems appear to be more vulnerable than other forest formations to diseases. In the last decades, severe decline events have severally impacted many riparian ecosystems worldwide (Nagel *et al*., 2015; Dunstan *et al*., 2016; Stamler *et al*., 2016; Bregant *et al*., 2023a). In particular, an increasing number of outbreaks caused by *Phytophthora* species have been reported on alders (Sims *et al*., 2015; Bregant *et al*., 2020). Although for many years the disease has been associated to the hybrid *P. ×alni*, currently over forty *Phytophthora* species have been isolated from declining alder tree across continents (Trzewik *et al*., 2016; Aday-Kaya *et al*., 2018; Sims *et al*., 2015; Bregant *et al*., 2020, 2023b). Several independent studies conducted in Europe and North America have shown that in the aetiology of the disease several *Phytophthora* species belonging to the ITS clade 2 are involved, such as *P. acerina*, *P.*

multivora, *P. plurivora* and *P. siskiyouensis* (Bregant *et al*., 2020, 2023b). In pathogenicity tests these species were shown to be aggressive alder pathogens, suggesting a primary role in the aetiology (Navarro *et al*., 2015; Seddaiu & Linaldeddu, 2020; Bregant *et al*., 2020, 2023b). Whereas the role of *Phytophthora* spp. in alder decline is well documented, their involvement in the emerging diseases affecting other riparian species remains understudied (Dunstan *et al*., 2016).

Given the alarming decline and mortality affecting riparian species in Italy and the still limited information on the pathogens involved, a study was conducted in forty-six riparian formations distributed from the Mediterranean to the alpine climate to isolate and characterize the main pathogens associated.

Materials & Methods

Surveys and sampling procedure

From November 2019 to March 2023, investigations were conducted in forty-six natural riparian systems and flooded wetland formations located in four Italian regions: Friuli Venezia Giulia, Sardinia, Trentino-Alto Adige and Veneto (Tab. 1). Survey areas ranged from 11 to 2050 m. a.s.l. and encompassed three different macroclimatic and floristic zones composed of various riparian formations: Mediterranean (site 1-6), temperate (site 7-16) and alpine (site 17-45) (Tab. 1).

In each site, plants were visually checked for the presence of *Phytophthora* disease symptoms on branches and stems (crown dieback, wilting shoots and bleeding cankers) and on the collar and root system (exudates, necrotic flames and root rots). In each site, a 50 m long transect was established to evaluate the disease incidence and mortality rate, estimated as reported in Bregant *et al*. (2023b).

Table 1. Study sites information and number of stem (s), root and rhizosphere (r) and leaf (l) samples used for *Phytophthora* isolation.

Study sites	Altitude (m. a.s.l.)	Climate	Geographic coordinates		Number of samples
1	150	Mediterranean	39.156918	8.904905	No(3r), Vt(2r), Qi(2r)
2	200	Mediterranean	39.092419	8.787526	No(2r), w(3l)
3	215	Mediterranean	39.093339	8.790907	Ag(2r), No(2r)
4	75	Mediterranean	41.098837	9.255745	Ag(4r), Sp(2r), w(10l)
5	50	Mediterranean	40.822237	8.886054	Tg(5r), Sp(5r), Pa(6r)
6	69	Mediterranean	40.845662	9.004490	Ag(3r, 2s)
7	72	Temperate	45.946786	13.558595	Ag(3r)
8	71	Temperate	45.953287	13.537717	Ag(5r, 1s), Ap(2r, 1s), Cb(1r), w(20l)
9	72	Temperate	45.954003	13.521796	Ag (3r)
10	69	Temperate	45.947667	13.528501	Ap(5r), Ac(4r), Ag(2r)
11	93	Temperate	45.977727	13.488724	Ap(2r)
12	400	Temperate	46.174719	13.633201	Ap(3r), Tc(2r), w(10l)
13	198	Temperate	46.200153	13.469431	Oc(2r), Ag(2r), Sa(1r)
14	371	Temperate	46.177453	13.537907	Ap(2r)
15	192	Temperate	45.841431	11.977090	Ag(2r)
16	11	Temperate	45.378485	11.970186	Ag(2r), Pn(2r), Pa(1r)
17	1029	Alpine	40.422742	8.995741	Ia(2r, 2s), Am(2r), Qp(3r), Tb(1r)

18	1145	Alpine	40.043707	9.206423	Ag(5r), Am(1r)
19	1134	Alpine	40.052639	9.254812	Pav(1r)
20	1631	Alpine	40.012519	9.299751	Ag (3r)
21	1493	Alpine	40.019592	9.278154	Ag(5r)
22	825	Alpine	39.921333	9.475791	Ag(4r,1s), w(20l)
23	997	Alpine	46.446888	12.727675	Ai (2r)
24	888	Alpine	46.505978	13.263095	Ai(8r,3s), w(20l)
25	916	Alpine	46.484227	13.674238	Ai(4r,1s)
26	610	Alpine	46.400660	13.419450	Ai(4r)
27	850	Alpine	46.582003	12.783226	Ai(2r)
28	1199	Alpine	45.941230	11.433070	Sal(3r), Fs(1r)
29	912	Alpine	46.462268	12.474627	Ai(10r, 4s), Ps(2r), Fs(2r), Bp(2r), w(50l)
30	1220	Alpine	46.467509	12.483365	Ai(6r,3s)
31	700	Alpine	46.473015	12.445977	Ai(4r, s2), w(50l)
32	1030	Alpine	46.471167	12.461170	Ai(3r,1s)
33	1300	Alpine	46.462513	12.504313	Ai(3r,2s)
34	1417	Alpine	46.492683	12.562043	Ai(20r, 13s), w(100l)
35	1600	Alpine	46.487834	12.565685	Av(8r), Ai(3r, 3s), w(50l)
36	1800	Alpine	46.476076	12.636677	Av(7r, 1s)
37	1785	Alpine	46.477789	12.593275	Av(11r, 3s), w(50l)
38	1475	Alpine	46.650916	12.447369	Ai(6r, 5s), w(50l)
39	1519	Alpine	46.648172	12.447797	Ai(4r, 1s)
40	1943	Alpine	46.666388	12.491231	Av(3r), Rf(2r), Sal(2r)
41	1319	Alpine	46.598536	12.469675	Jc(3r), Ps(2r)
42	1796	Alpine	46.406714	12.076475	Av(3r), Sal(2r)
43	1100	Alpine	46.813075	12.071103	Ai (2r, 2s)
44	2050	Alpine	46.884642	12.199748	Rf(3r), Av(2r)
45	1650	Alpine	46.158287	11.531815	Av(5r), Ai(2r)
46	1370	Alpine	46.856658	11.619803	Ai(3r)

* In brackets the number of samples collected from each plant species: *Acer campestre* (Ac), *Alnus glutinosa* (Ag), *Alnus incana* (Ai), *Acer monspessulanum* (Am), *Acer pseudoplatanus* (Ap), *Alnus viridis* (Av), *Betula pubescens* (Bp), *Carpinus betulus* (Cb), *Fagus sylvatica* (Fs), *Ilex aquifolium* (Ia), *Juniperus communis* (Jc), *Nerium oleander* (No), *Ostrya carpinifolia* (Oc), *Populus alba* (Pa), *Prunus avium* (Pav), *Populus nigra* (Pn), *Pinus sylvestris* (Ps), *Quercus ilex* (Qi), *Quercus pubescens* (Qp), *Rhododendron ferrugineum* (Rf), *Salix alba* (Sa), *Salix alpina* (Sal), *Salix purpurea* (Sp), *Taxus baccata* (Tb), *Tilia cordata* (Tc), *Tamarix gallica* (Tg) and *Viburnum tinus* (Vt).

A total of 260 symptomatic plants belonging to 27 different hosts were randomly sampled for *Phytophthora* isolation (Tab. 1). For each selected plant, about 300 g of rhizosphere (soil and fine roots) was collected around the collar. In addition, from some of the same plants, a bark sample was taken from the margin of an active bleeding canker on the stem or at the collar (51 samples).

The stream water of the sites: 2, 3, 8, 12, 22, 24, 29, 31, 34, 35, 37, 38 was monitored for *Phytophthora* through the baiting technique reported by Huberli *et al.* (2013). In particular, along each waterway 10 nylon mesh bags containing young *Quercus suber*, *Alnus* spp., *Fraxinus* spp. and *Sorbus aucuparia* young leaves as baits were positioned near the root systems of symptomatic trees. A total of 433 leaves were used as baits along the water streams.

Overall, 744 samples were processed for *Phytophthora* isolation. Once collected, the samples were sealed in plastic bags, labelled and processed in the laboratory within 24–48 h.

Isolates and morphology

Phytophthora isolates were obtained following the method described in Bregant *et al.,* (2020). In particular, root and rhizosphere samples were flooded with 2 L of distilled water in plastic boxes. Young cork oak and elder leaves were placed as baits on the water surface after 24h. Boxes were maintained at 15–20 °C under natural daylight for 3–5 days; after this period leaves showing dark spots were cut in small pieces (5 mm^2) and placed on Petri dishes containing potato dextrose agar (PDA 39 g/L, Oxoid Ltd., Basingstoke, UK) supplemented with 100 mL/L of carrot juice, 0.013 g/L of pimaricin and 0.05 g/L of hymexazol (PDA+) (Bregant *et al*., 2020).

Isolations were also directly attempted from tissue samples taken from active bleeding cankers. After removing the outer bark, small fragments were aseptically taken from the fresh lesion with a sterile scalpel and placed onto 90 mm Petri dishes containing PDA+. The dishes were incubated in the dark at 20 °C and examined every 6-12 h. Hyphal tips from the emerging colonies were sub-cultured on PDA and carrot agar (CA) and incubated at 20 °C in the dark.

Stream bags were collected after a week and transferred to the laboratory. Leaves showing necrotic spots were cleaned with distilled water, dried on sterile papers, cut into small fragments (5 mm^2) and used for *Phytophthora* isolations as described above.

Growth features in agar media, including surface and reverse colony appearance of all isolates were recorded after 7 and 14 days of incubation at 20 °C in the dark on PDA, CA and Pumpkin agar (PA; 200 g filtered pumpkin juice in 1 l of distilled water, 12 g technical agar Difco©). Morpho-biometric data of chlamydospores, gametangia and sporangia were recorded. To enhance sporangia production, CA and PA plugs (5 mm diameter) of each isolate were placed in Petri dishes containing unsterile pond water and three fine root alder samples (1 cm long). Petri dishes were incubated at 5, 10, 15, 20 and 25 °C in the dark and sporangia production was assessed every 12 h for 4 days. The mating behavior was also tested in dual culture on CA and PA, pairing two isolates of the same species together or with a heterothallic isolate of known mating type (A1 or A2) of *Phytophthora cambivora* [CB67 (A1); CB1 (A2)], *P. cinnamomi* [CBS 144.22 (A2)], *P. citrophthora* [CB314 (A1)] and *P. nicotianae* [CB315 (A1); CB316 (A2)] and an isolate of *Trichoderma atroviride*, as described by Brasier (1972). In addition, gametangia production was also verified placing a 5 mm diameter plug from the edge of an actively growing colony on CA and PA incubated at 10, 15, 20, 25 and 28 °C in the dark for 5-7 days in Petri dishes with 5 mL of unsterile pond water.

For three isolates belonging to the new species and other nine obtained from grey alder the effect of temperature on growth was tested *in vitro* on 90 mm CA Petri dishes incubated at 2, 5, 10, 15, 18, 20, 23, 25, 28, 30, 33, 35 and 38 °C (± 0.5 °C) in the dark. In more detail, agar plugs (5 mm diameter) taken from 4-day-old colonies, growing at 20 °C on CA, were placed top side down in the centre of

new media plates, with five replicates per isolate. After 96 h, the diameter of resulting colonies was measured twice at right angles on each plate and then averaged after subtraction of the original plug diameter.

For the new species, size and shape of fifty chlamydospores, hyphal swellings, sporangia, oogonia and anteridia were recorded for each isolate. Measurements and photos of the main morphological structures were taken at 200×, 400× and 600× magnification and recorded using the software Motic Images Plus 3.0 paired with a Moticam 10+ camera connected to a Motic BA410E microscope. Sporangia dimensions are presented as mean values ± standard deviation.

Representative isolates of each species were stored on PDA and CA slants under oil in the culture collection of Dipartimento Territorio e Sistemi Agro-Forestali, Università degli Studi di Padova. Representative cultures of the new species including the ex-type culture were deposited at the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, and nomenclatural data in Faces of Fungi (www.facesoffungi.org) and Index Fungorum (www.indexfungorum.org). The holotype was lodged with the herbarium of Westerdijk Fungal Biodiversity Institute as a dried culture on CA.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from mycelium of 4-day-old cultures grown on PDA at 20 °C using Instagene Matrix (BioRad Laboratories, Hercules, CA, USA). The entire internal transcribed spacer (ITS) region of the ribosomal DNA, including the 5.8S rRNA gene, was amplified and sequenced for all isolates using primers ITS1 and ITS4 to confirm the identification at species level (White *et al*., 1990).

For two isolates of the new species belonging to the ITS clade 6 (Yang *et al*., 2017), another two DNA regions, namely ß-tubulin (Btub) and cytochrome c oxidase subunit I (*cox*1), were amplified and sequenced using the primer-pairs TUBUF2/TUBUR1 and FM84/FM83 respectively (Martin & Tooley, 2003; Kroon *et al*., 2004). Polymerase chain reactions (PCR) were performed in 50 mL reaction mixtures using the GoTaq® Hot Start Green Master Mix (Promega, Milano, Italy) and a SimpliAmp Thermal Cycler (Thermo Fisher Scientific Inc.). Amplification conditions for the three regions were conducted as described in Bregant *et al*. (2023a).

PCR products were purified using a EUROGOLD gel extraction kit according to the manufacturer's instructions (EuroClone S.p.A., Pero, Italy). Both strands were sequenced by BMR Genomics DNA sequencing service (www.bmr-genomics.it). Sequences were edited with FinchTV v1.4.0 (Geospiza, Inc., http://www.geospiza.com/finchtv) and compared with sequences of ex-type

culture deposited in GenBank (http://blast.ncbi.nlm.nih.gov). New sequences were deposited in GenBank (Tabs 2 and 3). Alignments and trees are available in TreeBase (study ID 30787).

Phylogenetic analysis

ITS, Btub and cox1 sequences of two isolates of the new species obtained in this study and other four isolates available in GenBank were compiled in a dataset together with 252 sequences of other 63 *Phytophthora* species representative of the formally described species in the ITS clade 6 (including ex-type cultures) and some representative ex-type isolates belonging to the other eleven clades and for which DNA sequences are available in GenBank (Tab. 2). Sequences of *Nothophytophthora amphyginosa* were included as outgroup taxon.

Sequence alignments were performed with ClustalX v. 1.83 (Thompson *et al*., 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25%). Alignments were checked and edited with BioEdit Alignment Editor v. 7.2.5 (Hall, 1999). Phylogenetic analyses were done with MEGA-X 10.1.8 (Kumar *et al*., 2018). All gaps were included in the analyses. The best model of DNA sequence evolution was determined automatically by the software. Maximum likelihood (ML) analyses were performed with a neighbour-joining (NJ) starting tree generated by the software. A bootstrap analysis (1000 replicates) was used to estimate the robustness of nodes.

Table 2. *Phytophthora* isolates included in the Maximum Likelihood (ML) analyses. Ex-type cultures are given in bold typeface, and sequences obtained in this study are indicated in italics.

Species	**Code**	**Host**	**GenBank Accession Number**		
			ITS	**Btub**	***cox1***
Nothophytophthora amphigynosa	**BD268**	water	KY788382	KY788515	KY788473
Phytophthora alticola	**CBS 141718**	*Eucalyptus grandis*	KX247599	KX247592	KX247585
P. alpina	**OV3**	*Alnus viridis*	MT707332	MT729673	MT729668
P. amnicola	**CBS 131652**	water	JQ029956	JQ029952	MH477740
P. amnicola	VHS 19503	water	JQ029958	JQ029954	JQ029950
P. asparagi	**VHS 17644**	*Lomandra sonderi*	EU301168	JN547592	HQ012845
P. asparagi	VHS 17175	*Banksia media*	EU301167	JN547591	HQ012844
P. brassicae	**CBS 179.87**	*Brassica oleracea*	MG783384	AY564083	MH136857
P. balyanboodja	**CBS 143058**	soil	KJ372258	MN207267	MH477741
P. bilorbang	**CBS 161653**	*Rubus anglicandicans*	JQ256377	JQ256374	MH477742
P. bilorbang	SA146	*R. anglicandicans*	JN547624	JN547585	JN547646
P. boehmeriae	**CBS 291.29**	*Boehmeria nivea*	MG783382	MH493909	MH136852
P. borealis	**CBS 132023**	water	HM004232	JQ626615	MH136854
P. borealis	AKWA 57.2-0708	water	JQ626598	JQ626614	JQ626624
P. cambivora	**CBS 114087**	*Castanea sativa*	MG783387	MH493913	MH136860
P. capsici	**CBS 128.23**	*Capsicum annuum*	MG865467	MH493915	MH136863
P. castaneae	**P10187**	*Castanea crenata*	MG865470	MH493918	MH136866
P. chlamydospora	**P6133**	*Prunus* sp.	MG865471	MH493919	MH136867

P. chlamydospora	VHS 3753	soil	EU301160	HQ012878	JN547616
P. cinnamomi	**CBS 144.22**	*Cinnamomum burmannii*	MG865473	MH493920	MH136869
P. clandestina	**CBS 347.86**	*Trifolium subterraneum*	MG865477	MH493924	MH136873
P. cocois	**P19948**	*Cocos nucifera*	MG865478	MH493925	MH136874
P. condilina	**CBS 143059**	*Casuarina obesa*	KJ372262	MN207271	MH477744
P. cooljarloo	**CBS 143062**	*Hibbertia* sp.	HQ012957	MF326816	MH477745
P. crassamura	**PH138**	*Juniperus phoenicea*	KP863493	KX251202	KP863485
P. crassamura	CB267	*Cynara cardunculus*	MZ569853	OQ067252	OQ067256
P. debattistii	CBS 147721	*Alnus incana*	OP999674	OQ067250	OQ067254
P. debattistii	CB61	*A. incana*	OP999675	OQ067251	OQ067255
P. fluvialis	**CBS 129424**	water	MG865491	JN547595	MH136887
P. fluvialis	VHS 17350	water	EU593261	JN547593	JF701440
P. gemini	**CBS 123381**	*Zostera marina*	FJ217680	MF326818	HQ261440
P. gregata	**CBS 127952**	*Patersonia* sp.	MG865503	MH493945	MH477746
P. gregata	MJSP235	*Pinus radiata*	EU301172	JN547602	HQ012853
P. gibbosa	**CBS 127951**	*Acacia pycnantha*	MG865499	MH493942	MH136894
P. gibbosa	VHS 22008	*Grevillea* sp.	HQ012936	JN547597	HQ012849
P. gonapodyides	**P7050**	*Alnus* sp.	MG865501	MH493944	MH136896
P. gonapodyides	SLPA72	*Eucalyptus obliqua*	HQ012937	JN547598	HQ012850
P. heteromorpha	**CBS 148032**	*A. incana*	*OR575417*	*OR596854*	*OR596856*
P. heteromorpha	CB206	*A. incana*	*OR575418*	*OR596855*	*OR596857*
P. heteromorpha	RAS1	*Betula pendula*	HQ012964	JN547599	HQ012888
P. heteromorpha	SAM1	*Sambucus nigra*	JN547627	JN547660	JN547649
P. heteromorpha	SAM3	*S. nigra*	JN547628	JN547589	JN547650
P. heteromorpha	SAM4	*S. nigra*	JN547629	JN547590	JN547651
P. heveae	**CBS 296.29**	*Hevea brasiliensis*	MG865505	MH493947	MH136899
P. humicola	**P3826**	*Citrus* sp.	MK496519	MH493949	MK493476
P. infestans	**P1381**	*Solanum tuberosum*	MG865512	MH493954	MH136906
P. inundata	**CBS 216.85**	*Salix matsudana*	MG865516	MH493958	MH136910
P. irrigata	**P16861**	water	MG865520	MH493962	MH136914
P. kernoviae	**P19827**	*Fagus sylvatica*	MG865521	MH493963	MH136915
P. kwongonina	**CBS 143060**	*Banksia grandis*	JN547636	MF326824	MF326847
P. lacustris	**P245**	*S. matsundana*	JQ626605	JQ626619	MH136916
P. lacustris	HSA1959	water	HQ012956	JN547618	HQ012880
P. lilii	**CBS 135746**	*Lilium longiflorum*	MG865523	MH493965	MH136918
P. litoralis	**CBS 127953**	*Banksia* sp.	MG865526	MH493967	MH136921
P. litoralis	VHS 19173	*Banksia* sp.	EU869199	JN547610	HQ012865
P. meadii	**P19007**	*Hevea brasiliensis*	MG865529	MH493969	MH136924
P. megakarya	**CBS 238.83**	*Theobroma cacao*	HQ261610	KX251035	MH136929
P. megasperma	**CBS 402.72**	n/d	MG865535	MH493973	MH136930
P. megasperma	CB268	*Punica granatum*	OP999676	OQ067253	OQ067257
P. melonis	**CBS 582.69**	*Cucumis sativus*	MG865536	MH493974	MH136931
P. mississippiae	**P19994**	water	MG865542	MH493980	MH136935
P. mississippiae	57J4	water	KX251313	KF112853	KF112861
P. moyootj	**CBS 138659**	soil	KJ372256	KJ372303	MH477750
P. moyootj	DH103	water	KJ372255	KJ372301	KJ396700
P. multivesiculata	**CBS 545.96**	*Cymbidium* sp.	MG865544	MH493982	MH136937
P. nagaii	**CBS 133248**	*Rosa odorata*	MG865547	MN207274	MH136940
P. nicotianae	**P7661**	*Nicotiana tabacum*	MG865550	MH493985	MH136943
P. oreophila	**U11**	soil	MG542976	MG543037	MG543002
P. ornamentata	**CBS 140647**	*Pistacia lentiscus*	MG865556	MN207275	MH136947
P. ornamentata	PH153	*P. lentiscus*	KP863497	MN207276	KP863487
P. palmivora	**CBS 305.62**	*Areca catechu*	MG865559	MH493992	MH136949
P. personensis	**CBS 146549**	*Grevillea maccutcheonii*	EU301169	MF326805	HQ012877
P. pinifolia	**CBS 122924**	*Pinus radiata*	MG865566	MH493999	MH136958
P. pinifolia	CMW 26669	*P. radiata*	EU725807	JN935979	JN935961
P. plurivora	**CBS 124093**	*Fagus sylvatica*	MG865568	MH494001	MH136959
P. polonica	**P131445**	*Alnus glutinosa*	DQ396410	DQ399844	MH136960
P. pseudogregata	**CBS 149859**	*Juniperus communis*	OR167217	OR189513	OR189516
P. pseudogregata	CB308	*Rhododendron* sp.	OR167218	OR189514	OR189517
P. pseudorosacearum	**CBS 143061**	*Persoonia longifolia*	KJ372267	MN207277	MH477757

P. pseudosyringae	**CBS 111772**	*Quercus robur*	MG865574	MH494004	MH136966
P. psychrophila	**CBS 803.95**	*Q. robur*	MG865576	MH494005	MH136968
P. quercina	**CBS 784.95**	*Q. robur*	MG865578	MH494007	MH136970
P. ramorum	**CBS 101553**	*Rhododendron* sp.	MG865581	LC595884	MH136973
P. riparia	**CBS 132024**	water	MG865583	JQ626607	MH136975
P. riparia	VI 3-100B9F	water	HM004225	JQ626618	MH136975
P. rosacearum	**CBS 124696**	*Malus* sp.	EU925376	MN207278	MH477758
P. sansomeana	**CBS 117693**	*Glycine max*	MG865585	MH494012	MH136977
P. syringae	**CBS 110161**	*Syringa vulgaris*	MG865590	MH494016	MH136982
P. thermophila	**CBS 127954**	*Eucaliptus marginata*	MG865593	MH494019	MH136985
P. thermophila	VHS 16164	*Banksia grandis*	EU301158	JN547614	HQ012875
P. versiformis	**CBS 142005**	*Corymbia calophylla*	KX011279	MN207280	MH477760

Pathogenicity test

The pathogenicity of the new *Phytophthora* species and other nine species obtained from grey alder was tested on 4-year-old grey alder seedlings grown in plastic pots (10 cm diameter, 1 L volume). In the assay, five seedlings were used for each isolate whereas four seedlings were used as control. Inoculations were performed at the collar after that surface (outer bark) had been disinfected with 70% ethanol. A small piece of outer and inner bark (5 mm diameter) was removed with a flamed cork borer and replaced with an agar-mycelium plug of the same size taken from the margin of an actively growing colony on PDA. The inoculation site was covered with cotton wool soaked in sterile water and wrapped in a piece of aluminium foil. Controls were inoculated with a sterile PDA plug applied as described above. All inoculated seedlings were kept in field conditions at 10 to 33 °C and watered regularly for 30 days. At the end of the experimental period, seedlings were checked for the presence of disease symptoms; the outer bark was carefully removed with a scalpel and the length of necrotic lesion surrounding each inoculation point was measured.

Furthermore, the pathogenicity of these species was tested also on spring young leaves of grey alder taken from 4-year-old seedlings. The leaves were initially flooded in distilled water, placed in Petri dishes (12 cm diam.) containing a sheet of sterile filter paper soaked in distilled water and then inoculated by wound (puncture performed with a sterile needle) with a 5 mm diameter mycelium plug obtained from 4-day colonies on PDA. Five leaves were inoculated with each isolate, and five were used as control, inoculating a sterile PDA plug. The plug of agar-mycelium was placed (with the side containing the mycelium in contact with the leaf) in the centre of the upper page, in correspondence to the wound. The plates were kept in controlled conditions at 20 °C. After 72 hours the size of the necrotic area on the leaf caused by each pathogen on the leaf was measured using the Assess v. 2.0 (Lamari, 2002).

For both assays re-isolation of isolates was attempted by transferring 5 pieces of symptomatic tissues taken around the margin of the necrotic lesions onto PDA+. Growing colonies were sub-

cultured onto CA and PDA, incubated in the dark at 20 °C and identified by morphological and molecular analysis (ITS region).

Statistical analyses

Pathogenicity assay data were checked for normality, then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using Fisher's least significant differences multiple range test ($p = 0.05$) after one-way ANOVA using XLSTAT 2016 software (Addinsoft).

Similarities in fungal taxonomic assemblage among climatic areas were summarized in Venn diagrams using GeneVenn software (https://www.bioinformatics.org/gvenn/) to generate the diagram (https://www.bioinformatics.org/gvenn/) and reconstructing it in Canva (https://www.canva.com/).

Results

Field survey

Field surveys conducted in 46 sites distributed in four different Italian regions showed the widespread presence of *Phytophthora*-related disease symptoms on twenty-seven plant species typical of riparian systems and wetlands. Similar symptoms were observed in all riparian formations from Mediterranean areas to the subalpine belt (Fig. 1). Among the different sites, the estimated disease incidence ranged from 30 to 100% with a mortality rate of 5-70%. *Alnus* spp. showed the highest mortality rate.

Affected plants showed an extremely complex symptomatology, including primarily crown dieback symptoms such as transparency and wilting foliage, shoot blight and abundant production of epicormic shoots on stem and branches (Fig. 2). Extensive aerial bleeding cankers were often observed on the whole canopy. Bleeding lesions progressively girdled the entire circumference of stem and branches, causing crown dieback, dying foliage and sudden death. At the same time, young foliage of several mountain species often displayed necrotic spots and wilting, suggesting aerial infections of *Phytophthora* (Fig. 2). The sudden death of the crown was the result of an extensive or total destruction of the root system (Fig. 2).

Figure 1. Overview of different declining riparian systems included in the study: Mediterranean riparian habitats dominated by *Nerium oleander*, *Salix* spp. and *Populus* spp. (a-c), temperate systems populated by common alder and maples (d-f); riparian vegetation of *Alnus* spp., *Salix* spp. and *Rhododendron* spp. along alpine streams (g-i).

Figure 2. Disease symptoms observed along streams and rivers: shoot blight and death foliage of *Alnus viridis* (a,b), epicormic shoots and declining canopy of *Alnus incana* (c), *Populus alba* (d) and *Tamarix gallica* (e), necrotic beech leaves naturally flooded in streams (f); bleeding stem cankers and wood necrosis on *Acer campestre* (g), *Alnus incana* (h,i), *Fagus sylvatica* (j) and *Salix alpina* (k). Collar and root rot symptoms on *Prunus avium* (l), *Acer pseudoplatanus* (m) and *Alnus* spp. (n-p).

Phytophthora diversity

A total of 691 *Phytophthora* isolates were obtained from the 744 samples processed (positivity 92%). Based on colony appearance, sporangia morphology and sequence data of the ITS region, 20 known species belonging to seven ITS phylogenetic clades were identified: *P. plurivora* (202 isolates), *P. gonapodyides* (156), *P. pseudosyringae* (84), *P. lacustris* (57), *P. acerina* (31), *P. idaei* (30), *P alpina* (20), *P. pseudocryptogea* (19), *P. cambivora* (13), *P. pseudotsugae* (13), *P. cactorum* (9), *P. hydropathica* (6), *P. pseudogregata* (6), *P. debattistii* (4), *P. multivora* (4), *P. cinnamomi* (3), *P. bilorbang* (2) and *P. crassamura* (2), *P. ilicis* (2) and *P. inundata* (2) (Fig. 3). In addition, 26 isolates on the basis of morphological features and DNA sequence data could not be assigned to any formally described species of *Phytophthora*. These isolates were obtained from rhizosphere soil samples of *Alnus incana* and *Pinus sylvestris* and showed identical ITS, Btub and *cox*1 sequences.

Table 3. Number of isolates of each *Phytophthora* species obtained from stem, rhizosphere and water samples.

Species	ITS clade	Accession number	Number of isolates			Total	Number of sites
			Stem	Rhizosphere	Water		
P. acerina	2	OR575409	1	18	12	31	8
P. alpina	1	OR575410	2	12	6	20	3
P. bilorbang	6	OR575411	-	2	-	2	2
P. cactorum	1	OR575412	1	5	3	9	3
P. cambivora	7	OR575413	2	3	8	13	2
P. cinnamomi	7	OR575414	-	3	-	3	1
P. crassamura	6	OR575415	-	2	-	2	1
P. debattistii	6	OP999674	3	1	-	4	2
P. gonapodyides	6	OR575416	3	24	129	156	16
P. heteromorpha	6	OR575417	-	6	20	26	2
P. hydropathica	9	OR575419	-	2	4	6	2
P. idaei	1	OR575420	-	5	25	30	2
P. ilicis	3	OR575421	2	-	-	2	1
P. inundata	6	OR575422	-	2	-	2	1
P. lacustris	6	OR575423	-	25	32	57	11
P. multivora	2	OR575424	-	4	-	4	1
P. plurivora	2	OR575425	11	82	109	202	25
P. pseudocryptogea	8	OR575426	2	12	5	19	5
P. pseudogregata	6	OR575427	-	6	-	6	2
P. pseudosyringae	3	OR575428	4	46	34	84	15
P. pseudotsugae	1	OR575429	-	5	8	13	2

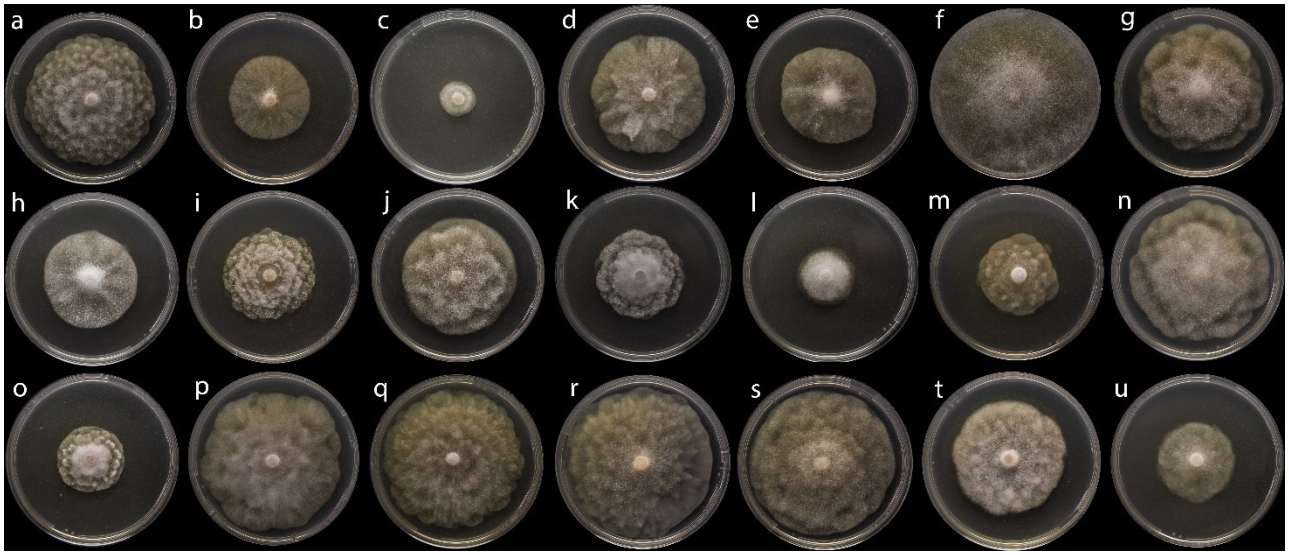

Figure 3. - Colony morphology of *Phytophthora acerina* (a), *P. alpina* (b), *P. bilorbang* (c), *P. cactorum* (d), *P. cambivora* (e), *P. cinnamomi* (f), *P. crassamura* (g), P. debattistii (h), *P. gonapodyides* (i), *P. heteromorpha* (j), *P. hydropathica* (k), *P. idaei* (l), *P. ilicis* (m), *P. inundata* (n), *P. lacustris* (o), *P. multivora* (p), *P. plurivora* (q), *P. pseudocryptogea* (r), *P. pseudogregata* (s), *P. pseudosyringae* (t), *P. pseudotsugae* (u) on Pumpkin agar (PA) after 7 days at 20 °C in the dark.

Overall, 21 *Phytophthora* taxa belonging to phylogenetic clades 1, 2, 3, 6, 7, 8 and 9 were obtained from rhizosphere or bleeding cankers at 45 of the 46 riparian sites monitored, whereas 13 *Phytophthora* taxa belonging to phylogenetic clades 1, 2, 3, 6, 7, 8 and 9 were baited along the 12

streams (Tab. 3). Among the different *Phytophthora* communities, *P. plurivora* was the dominant one, it was isolated in all four investigated regions from 25 sites and 13 plant hosts. *Phytophthora gonapodyides* and *P. lacustris* were the other two dominant species, they were isolated mainly in waterways in all three climatic areas monitored (Fig. 4). In addition, other 6 species (*P. acerina*, *P. alpina*, *P. cactorum*, *P. cambivora*, *P. pseudocryptogea* and *P. pseudosyringae*) were isolated from all three types of samples, although their distribution and host range appeared more fragmented. The remaining species were isolated preferentially from only one or two substrates (Tab. 4).

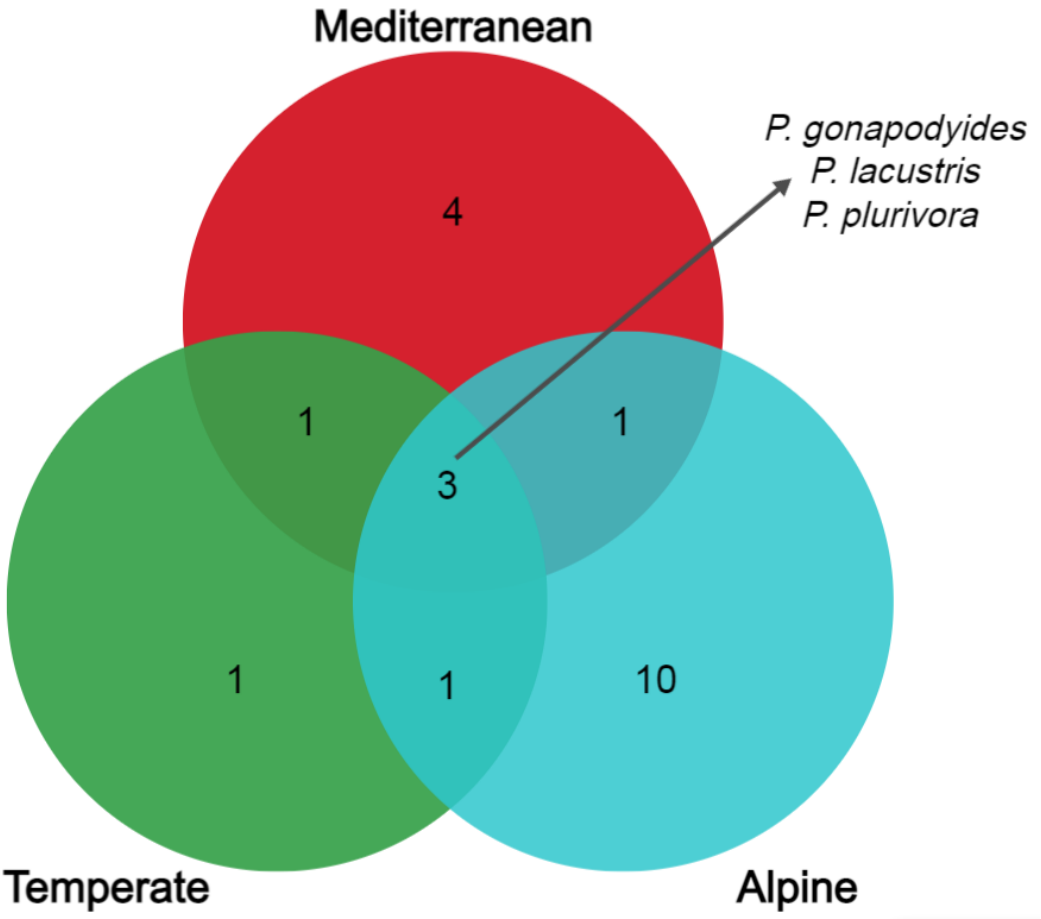

Figure 4. Venn diagrams illustrating the number of unique and shared *Phytophthora* species among the three climatic regions.

Table 4. Number of *Phytophthora* isolates obtained from each plant species.

	***Phytophthora* species**																				
Host species	**ACE**	**ALP**	**BIL**	**CAC**	**CAM**	**CIN**	**CRA**	**DEB**	**GON**	**HET**	**HYD**	**IDA**	**ILI**	**INU**	**LAC**	**MUL**	**PLU**	**PSC**	**PSG**	**PSY**	**PST**
Acer campestre	1*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2*	1*	-	-	-	-
Acer monspsessulanum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-
Acer pseudoplatanus	8	-	-	-	-	-	-	-	-	-	-	-	-	-	13	2*	3	-	-	-	-
Alnus glutinosa	-	-	1	-	-	-	-	-	9	-	2	-	-	-	-	-	11	3	-	14	-
Alnus incana	8	-	-	6	5	-	-	4	7	5*	-	5	-	-	-	-	59	6	-	10	-
Alnus viridis	-	14	-	-	-	-	-	-	3*	-	-	-	-	-	-	-	2	-	5	11	5*
Betula pubescens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-
Carpinus betulus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
Fagus sylvatica	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-
Ilex aquifolium	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-
Juniperus communis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	1	-
Nerium oleander	-	-	-	-	-	-	1*	-	-	-	-	-	-	-	1	-	-	4	-	-	-
Ostrya carpinifolia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	1	-	-	-	-
Populus alba	-	-	1*	-	-	-	-	-	4*	-	-	-	-	-	-	-	-	1	-	-	-
Prunus avium	-	-	-	-	-	-	-	-	1*	-	-	-	-	-	-	-	-	-	-	-	-
Populus nigra	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-
Pinus sylvestris	-	-	-	-	-	-	-	-	-	1*	-	-	-	-	-	-	3*	-	-	-	-
Quercus ilex	-	-	-	-	-	3	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Quercus pubescens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-
Rhododendron ferrugineum	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	3	-
Salix alba	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-
Salix alpina	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	4	-
Salix purpurea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3*	-	-	-	-
Taxus baccata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
Tilia cordata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2*	-	-	-	-
Tamarix gallica	-	-	-	-	-	-	-	-	3*	-	-	-	-	2*	3	-	-	-	-	-	-
Viburnum tinus	-	-	-	-	-	-	1*	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Phytophthora species: *P. acerina (*ACE), *P. alpina* (ALP), *P. bilorbang* (BIL), *P. cactorum* (CAC), *P. cambivora* (CAM), *P. cinnamomi* (CIN), *P. crassamura* (CRA), P. *debattistii* (DEB), *P. gonapodyides* (GON), *P. heteromorpha* (HET), *P. hydropathica* (HYD), *P. idaei* (IDA), *P. ilicis* (ILI), *P. inundata* (INU), *P. lacustris* (LAC), *P. multivora* (MUL), *P. plurivora* (PLU), *P. pseudocryptogea* (PSC), *P. pseudogregata* (PSG), *P. pseudosyringae* (PSY) and *P. pseudotsugae* (PST). *New host-pathogen associations.

Phylogenetic analyses

Phylogenetic relationships among the representative isolates belonging to the new species obtained in this study and the other formally described *Phytophthora* species belonging to clade 6 and some ex-type isolates of the other 11 clades were clarified using a multilocus analysis based on concatenated sequences of nuclear (ITS and Btub) and mitochondrial (*cox*1) DNA regions. Fragments of approximately 800, 920 and 1020 bp were obtained for ITS, Btub and *cox*1 regions, respectively.

The ML evolutionary reconstruction allowed 33 well supported terminal clades within the ITS clade 6 to be recognized, corresponding to 33 species (Fig. 5).

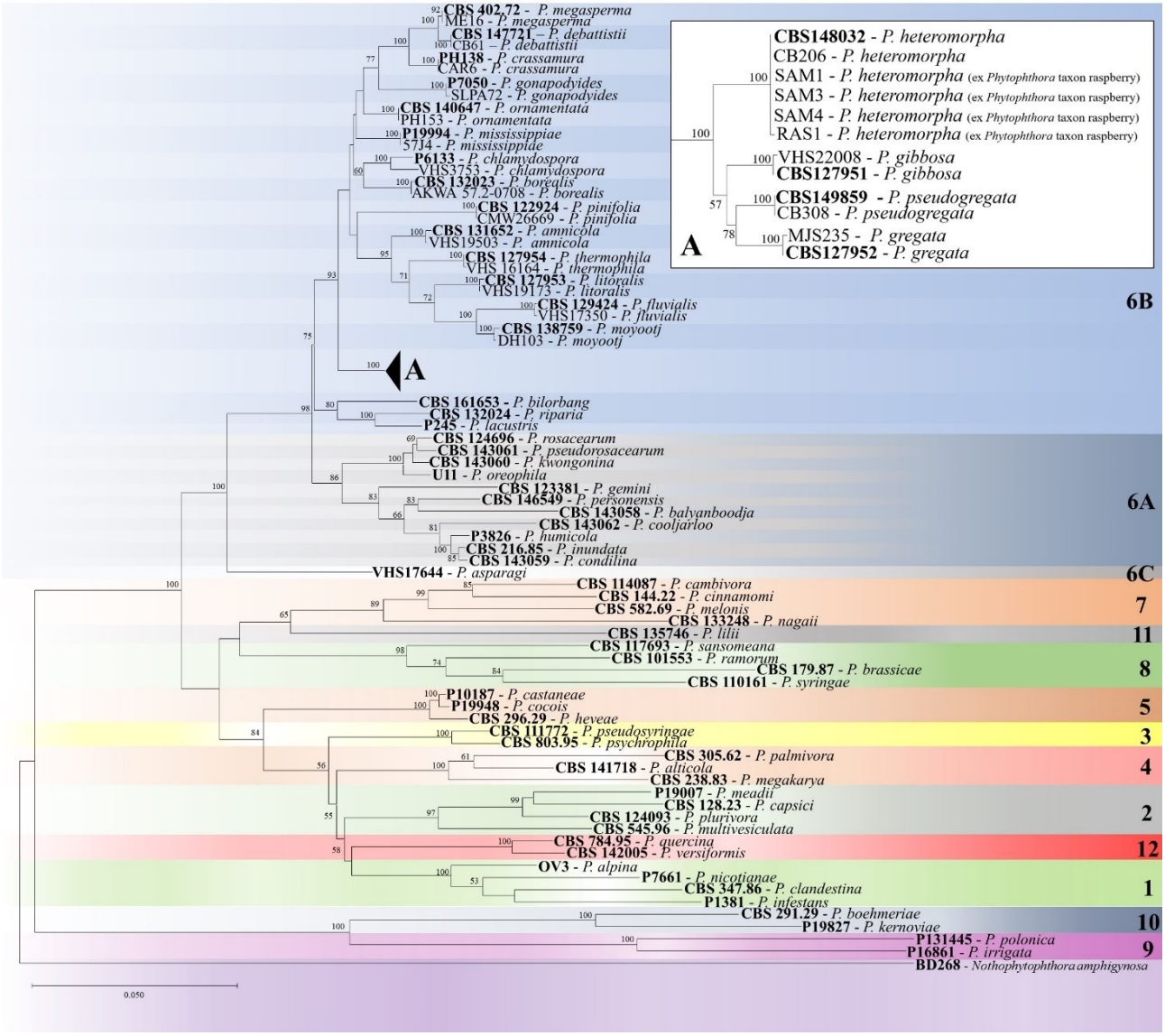

Figure 5. Maximum likelihood tree obtained from concatenated ITS, Btub and *cox*1 sequences of 64 *Phytophthora* species. Data are based on the General Time Reversible Model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap support values in percentage (1000 replicates) are given at the nodes. Ex-type cultures are in bold.

The two isolates obtained in this study clustered together in a well-supported terminal clade (ML bootstrap = 100%) within the subclade 6b and were considered to represent a new species formally described here as *Phytophthora heteromorpha* sp. nov. (Fig. 5). In the same terminal clade also some isolates available on GenBank clustered, from Europe, previously designated as *Phytophthora* taxon raspberry. These isolates are not linked to any formal description.

Phylogenetically, *Phytophthora heteromorpha* is closely related to *P. gibbosa*, *P. gregata* and *P. pseudogregata* but can be distinguished by differences in ITS, Btub and *cox*1 sequences.

Taxonomy

Phytophthora heteromorpha Bregant & Linaldeddu, sp. nov.

Index Fungorum number: IF901135; FoF number: FoF14923; Mycobank: MB850231

Etymology: the name refers to the different morphological appearance of the sporangia.

Description: Sporangia were abundantly produced on CA and PA plugs flooded in unsterile pond water after 48 h of incubation at 25 °C on simple sporangiophores. They were persistent, non-papillate, terminal, pyriform (40%), ovoid (35%) and ellipsoid (25%) (Figure 6 d-f). Sporangia average 60.5 ± 10.6 × 36.3 ± 5.8 µm (overall range 41.0 – 99.5 × 26.9 - 52.5 µm), length/breadth ratio 1.70 ± 0.21 µm (n=50). Sporangia usually proliferated externally, sometimes internally in an extended and nested way (Fig. 6 h-j). Zoospores were abundantly released in liquid cultures after 48 h at 25 °C in the dark (Fig. 6 h). Encysted zoospores were frequently observed inside sporangia in all isolates; cists germinated commonly inside sporangia releasing a tube and secondary microsporangia (Fig. 6 k-o). Zoospores of *P. heteromorpha* averaged 8.6–19.2 µm wide (av. 13.2 ± 3.7 µm, n=50). They were globose to reniform whilst release, spherical on encystment (Fig. 6 p). Cysts usually germinated in water by forming a tube, evolving in hypha (Fig 6 q-s). Hyphal swellings irregular or globose, terminal or intercalary catenulate, were produced in solid culture and unsterile pond water after 72 h in the dark (Figure 6 t,u). Terminal chlamydospores are occasionally observed in liquid culture after 72-96 h of incubation at 15 °C in the dark, absent in solid media, average diameter 24.1 ± 3.4 µm (overall range 18.3 – 29.1, n=50) (Fig. 6 v).

None of the *P. heteromorpha* isolates tested produced gametangia in single solid culture or when paired with other isolates of *P. heteromorpha*, A1 and A2 tester strains of *P. cambivora* and *P. cinnamomi*, *P. citrophthora* and *P. nicotianae* or with an isolate of *Trichoderma atroviride*. Also, none of the isolates stimulated the formation of oogonia in the A1 or A2 tester strains. All isolates formed rarely gametangia in single CA culture when flooded in unsterile pond water after 72-96 of incubation at 25 °C in the dark. Oogonia diameter was 33.2 ± 4.3 µm with a total range of 23.0–42.2 µm (n=50). Oospores were aplerotic and measured 30.1 ± 3.8 µm in diameter with relatively thick oospore walls (on av. 3.0 ± 0.6 µm). The antheridia were mostly paragynous (71%), sometimes amphyginous (29%) and measured 15.0 ± 1.9 × 10.9 ± 1.9 µm (n=50), one attached per oogonia (Fig. 6 w,x).

Cultural characteristics: colony growth without distinct pattern and irregular border on PDA, radial on MEA and rosaceous to stellate on CA. On PDA colony growth was slow, whereas on MEA and CA colony reached 67- and 72-mm diameter in 7 days at 23 °C, respectively.

Cardinal temperatures for growth: minimum <2 °C, maximum 35 °C and optimum 23 °C. Isolates failed to grow at 38 °C and mycelium did not resume growth when plates were moved to 20 °C (Fig. 7).

Material examined: Italy, Lorenzago di Cadore, isolated from rhizosphere of declining *Alnus incana*, 26 Jul 2020, collected and isolated by Carlo Bregant, HOLOTYPE CBS H-24775, a dried culture on CA, culture ex-holotype CB74 = CBS 148032. ITALY: Lorenzago di Cadore, isolated from rhizosphere of declining *Alnus incana*, 15 April 2021, collected and isolated by Carlo Bregant, isolate CB206. ITALY: Lorenzago di Cadore, isolated from rhizosphere of symptomatic *Pinus sylvestris*, 15 April 2021, collected and isolated by Carlo Bregant, isolate CB184.

Notes – Isolates of *P. heteromorpha* share some of the common characters of the species belonging to subclade 6b (Jung *et al*., 2011; Bregant *et al*., 2023a). However, *P. heteromorpha* can easily be distinguished from the closest species based on a combination of genetic and morphological differences (Tabs 5 and 6). *Phytophthora heteromorpha* differs from the sister species *P. gibbosa*, *P. gregata* and *P. pseudogregata* by shape and size of sporangia, chlamydospores and an abundant formation of encysted zoospores with high germination rate inside sporangia and production of microsporangia. *Phytophthora heteromorpha* differs from *P. gregata* and *P. pseudogregata* also for the breeding systems, as well as a total of 27 and 33 fixed nucleotide differences in the ITS, Btub, and cox1 sequences, respectively and from *Phytophthora gibbosa* for the smooth wall oogonia and bigger size of sporangia as well as 46 fixed polymorphisms in the three DNA regions studied (Tab. 5).

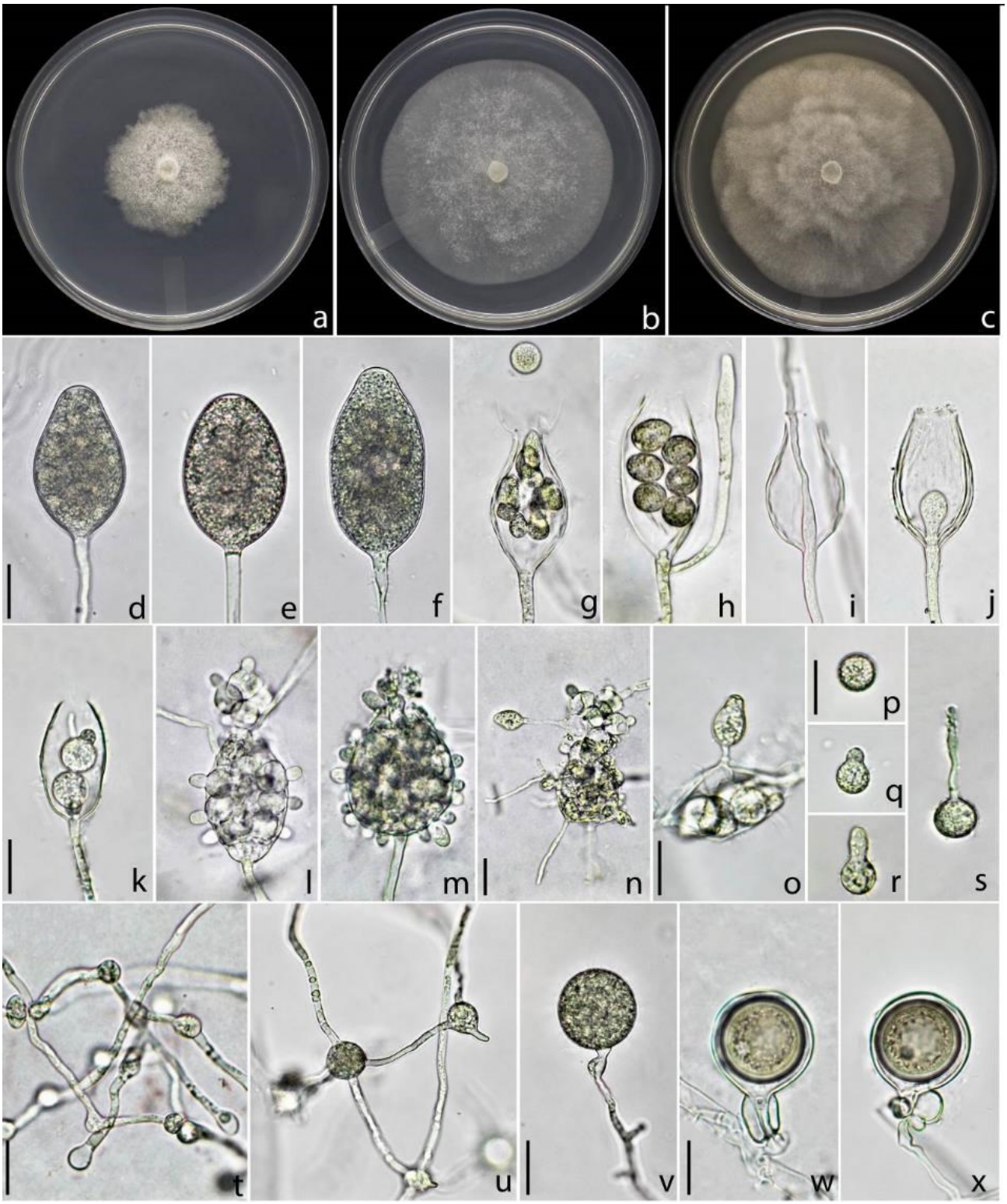

Figure 6. Colony morphology of *Phytophthora heteromorpha* (CB74) after 7 days growth at 20 °C on PDA (a), MEA (b) and CA (c). Non-papillate persistent sporangia (d-g); external proliferation (h); internal nested and extended proliferation (i,j); encysted zoospores with germ tube inside sporangia (k-m); zoospores germinating within the sporangium and producing microsporangia (n,o); zoospore after rounding up (p); zoospores germinating in unsterile water (q-s). Intercalary globose hyphal swellings (t,u); terminal chlamydospore (v). Oogonia with amphygynous (w) and paragynous antheridia (x). Scale bars = 20 µm.

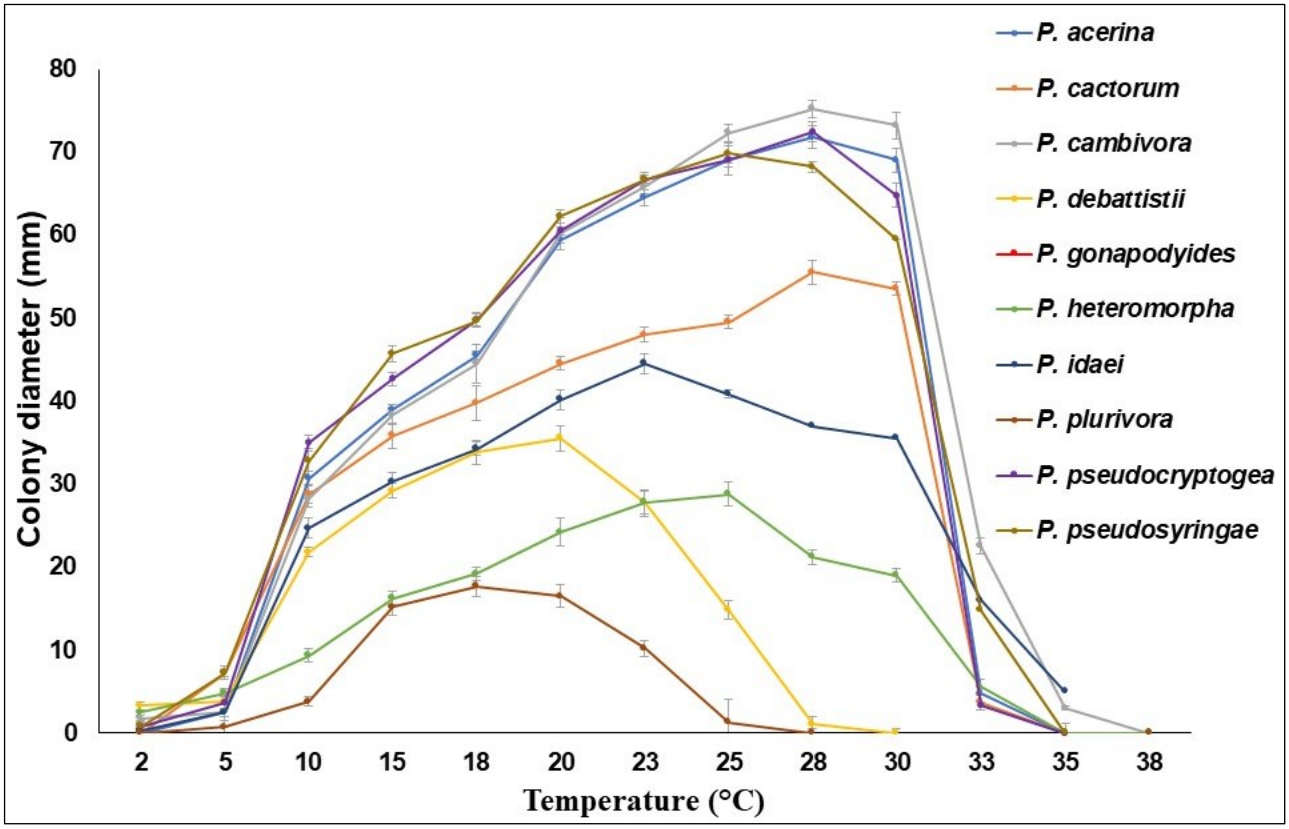

Figure 7. Mean colony diameter (± standard deviation) of ten *Phytophthora* species obtained from *Alnus incana*: *P. acerina* (CB340), *P. cactorum* (CB341), *P. cambivora* (CB67), *Phytophthora debattistii* (CB83), *P. gonapodyides* (CB342), *P. heteromorpha* (CBS 148032), *P. idaei* (CB343), *P. plurivora* (CB344), *P. pseudocryptogea* (CB345), *P. pseudosyringae* (CB346) after 96 h on CA in the dark at different temperatures.

Table 5. Polymorphic sites from aligned ITS, ß-tubulin and *cox1* sequence data showing interspecific variation of the new species described and the ex-type sequences of other three closely *Phytophthora* taxa from Subclade 6b.

ITS

***Phytophthora* species, isolate**	165	457	459	531	675	677	694	719	721	723	744
P. gibbosa (CBS 127951)	T	C	G	A	A	A	G	T	G	C	T
P. gregata (CBS 127952)	T	C	G	G	G	G	A	T	G	T	T
P. heteromorpha (CB74)	C	C	G	G	A	A	A	C	G	T	C
P. pseudogregata (CB234)	T	T	A	G	G	G	A	T	A	T	T

ß-tubulin

***Phytophthora* species, isolate**	024	204	210	480	765	864
P. gibbosa (CBS 127951)	S	R	C	G	C	A
P. gregata (CBS 127952)	C	G	Y	A	C	G
P. heteromorpha (CB74)	C	G	C	G	T	G
P. pseudogregata (CB234)	C	G	C	G	C	G

cox1

***Phytophthora* species, isolate**	046	127	148	163	223	235	289	344	385	397	409	436	463	466	496	526	532	535	538	541	547	583	640	700	733	757	787	808	835	847	907	922	925	943	964	970	1071	1075
P. gibbosa (CBS 127951)	C	G	T	T	T	A	A	T	T	T	T	G	C	A	T	T	T	T	G	A	T	T	T	A	C	A	T	A	A	C	A	T	T	T	T	T	A	C
P. gregata (CBS 127952)	T	G	A	T	T	T	C	A	T	T	T	A	T	G	C	A	C	T	G	A	C	C	A	T	T	A	C	T	A	T	T	T	C	A	T	C	G	T
P. heteromorpha (CB74)	T	A	A	C	A	T	T	A	C	C	T	A	G	G	C	A	A	G	G	G	C	C	A	T	T	G	A	T	T	T	T	C	T	A	C	T	A	T
P. pseudogregata (CB234)	C	G	T	T	T	T	T	A	T	T	A	A	A	A	C	T	A	T	A	A	T	T	A	A	T	A	T	A	A	T	T	C	T	A	C	C	G	C

Table 6. Morphological characters, morphometric data and temperature-growth relationships of *Phytophthora heteromorpha* and closely related species in subclade 6b.

	P. heteromorpha	***P. gibbosa***	***P. gregata***	***P. pseudogregata***
Number of isolates examined	3	Jung *et al*., 2011	Jung *et al*., 2011	Bregant *et al*., 2023a
Sporangia	Pyriform to ovoid and ellipsoid, nonpapillate	Ovoid, ellipsoid, nonpapillate, some semipapillate	Ovoid, limoniform, obpyriform, nonpapillate	Ovoid to obpyriform, sometimes ellipsoid, nonpapillate, some semipapillate
Length × breadth mean (µm)	60.5 ± 10.6 × 36.3 ± 5.8	48.8 ± 9.6 × 30.8 ± 5.4	51.0 ± 13.8 × 30.5 ± 5.9	50.3 ± 6.5 × 29.9 ± 3.8
Total range	41.0 – 99.5 × 26.9 - 52.5	24.8–71.1 × 17.4–48.0	25.7–102.3 × 14.8–50.7	32.1 – 65.1 × 22.1 – 38.4
Length/Breadth ratio	1.70 ± 0.21	1.58 ± 0.15	1.67 ± 0.32	1.68 ± 0.21
Microsporangia	Abundant	-	-	Not observed
Proliferation	Mostly external, sometimes internal, both extended and nested way	Internal extended, external, never nested	Internal extended and nested, never external, sporangiophore partly branching inside empty sporangium	Mostly external, sometimes internal, mostly extended and rarely nested
Hyphal swellings	Irregular or globose, terminal or intercalary, catenulate	Subglobose, elongated, never catenulate	Globose, elongated, angular, partly catenulate	Globose to subglobose, mostly intercalary catenulate, rarely terminal
Chlamydospores	Terminal chlamydospores	Not observed	Not observed	Not observed
Mean diameter (µm)	24.1 ± 3.4 µm	-	-	-
Diameter range (µm)	18.3 – 29.1	-	-	-
Breeding systems	Homothallic or self-fertile	Homothallic	Homothallic or self-fertile	Homothallic
Oogonia	Smooth	Ornamented, smooth	Smooth	Smooth
Mean diameter (µm)	33.2 ± 4.3	38.1 ± 5.4	36.8 ± 4.1	33.2 ± 3.4
Diameter range (µm)	23.0–42.2	27.0–49.9	23.9–50.9	26.9-41.6
Oospores	Aplerotic	Always aplerotic	Usually aplerotic	Aplerotic
Mean diameter (µm)	30.1 ± 3.8	31.4 ± 4.6	31.6 ± 4.0	29.1 ± 3.7
Total range (µm)	21.2–43.3	18.9–39.4	21.4–45.3	20.5-37.8
Wall thickness (µm)	2.78 ± 0.55	3.17 ± 0.69	2.65 ± 0.81	2.18 ± 0.71
Antheridia	Mostly paragynous (71%), sometimes amphyginous (29%)	Amphigynous	Mostly paragynous	Mostly amphygynous (58%), occasionally paragynous (42%)
Length × breadth mean (µm)	15.0 ± 1.9 × 10.9 ± 1.9	13.6 ± 2.4 × 14.0 ± 2.0	17.1 ± 3.0 × 11.0 ± 1.8	16.2 ± 2.7 × 12.5 ± 2.2
Total range (µm)	10.9-18.9 × 6.9-14.2	10.6–24.9 × 7.6–17.8	10.6–24.9 × 7.6–17.8	11.7-23.3 × 9.0-17.9
Maximum temperature (°C)	35 ≤ 38	32.5 ≤ 35	32.5 ≤ 35	32
Optimum temperature (°C)	23	30	25	23

Pathogenicity tests

All *Phytophthora* species proved to be pathogenic on grey alder. At the end of the experimental period, all seedlings displayed dark brown inner bark lesions that spread up and down from the inoculation site (Fig 6). The average lesion length differed significantly among species (Tab. 7). In the first assay conducted inoculating the stem of the seedlings the lesions caused by *P. cambivora* and *P. plurivora* were significantly larger than those caused by other species. Also *P. acerina*, *P. cactorum* and *P. pseudosyringae* caused very large necrotic lesions. In addition, seedlings inoculated with *P. cambivora*, *P. plurivora* and *P. pseudosyringae* displayed stem exudates, congruent with field observations. Although with a shorter length, the new species also caused significantly necrotic lesions on the stem.

Table 7. Mean lesion size ± standard deviation caused by each *Phytophthora* species on grey alder leaves (*) and seedlings (**).

Species	Isolate	Mean lesion area* (mm^2)	Mean lesion length** (mm)	Re-isolation (%)
P. acerina	CB340	23.1 ± 23.7c	33 ± 5b	100
P. cactorum	CB341	142.8 ± 208bc	23 ± 3c	100
P. cambivora	CB67	134.2 ± 121.2bc	52 ± 3a	100
P. debattistii	CB83	25.3 ± 23.4c	18 ± 7cde	100
P. gonapodyides	CB342	29.1 ± 14.1c	13 ± 3e	100
P. heteromorpha	CBS 148032	51.1 ± 77.2c	20 ± 4cd	100
P. idaei	CB343	428.9 ± 389.1a	18 ± 3cd	100
P. plurivora	CB344	379.8 ± 299.1ab	46 ± 3a	100
P. pseudocryptogea	CB345	100.6 ± 55.1c	15 ± 2de	100
P. pseudosyringae	CB346	607.3 ± 416.3a	22 ± 9cd	100
Control	-	-	5 ± 3f	-

After 72 h, all leaves inoculated with *Phytophthora* spp. showed dark brown necrosis, which spread on the surface of the leaf from the inoculation site (Fig. 8). Necrotic lesion area differed statistically between the ten *Phytophthora* species. The lesions caused by *P. pseudosyringae*, *P. idaei* and *P. plurivora* were significantly larger than those caused by other species (Tab. 7 and Fig. 8). *Phytophthora acerina*, *P. gonapodyides* and the new species caused only a small lesion confined to the inoculation site.

Control seedlings and leaves inoculated with sterile PDA plugs remained symptomless, only a small light brown discoloration was observed restricted to the inoculation point on seedlings. All ten species were successfully re-isolated from the margin of necrotic leaf and bark, thus fulfilling Koch's postulates.

Figure 8. Symptoms observed on grey alder leaves (after 72 h) and seedlings (after 30 days) inoculated with *Phytophthora acerina* (a-a2), *P. cactorum* (b-b2), *P. cambivora* (c-c2), *P. debattistii* (d-d2), *P. gonapodyides* (e-e2), *P. heteromorpha* (f-f2), *P. idaei* (g-g2), *P. plurivora* (h-h2), *P. pseudocryptogea* (i-i2), *P. pseudosyringae* (j-j2). Control (k-k2).

Discussion

Field surveys conducted in four Italian regions over a four-year period showed a complex of pathogenic *Phytophthora* species associated with leaf and shoot blights, bleeding cankers and root rot symptoms on twenty-seven plant species typical of riparian vegetation. The complex of symptoms observed were compatible with both air and soil-borne *Phytophthora* infections. Until now, knowledge on *Phytophthora* diversity and distribution across riparian vegetation in Italy has been

very limited. The extensive field surveys conducted in this study highlight that the severe disease outbreaks and mortality affecting alders along riparian habitats (Bregant *et al*., 2020) are also currently affecting several other woody plant species belonging to the *Apocynaceae*, *Betulaceae Fagaceaee*, *Salicaceae*, *Sapindaceae* and *Tamaricaceae* families along river and streams from the Mediterranean to the alpine riparian areas. Alder decline has been associated for a long time, especially in central Europe, to the attack of the hybrid *Phytophthora ×alni* (Bjelke *et al*., 2016; Brasier *et al*., 2004). However, recent investigations in Austria, Czech Republic, Italy, Portugal and Slovakia, as well as this study, have contributed to better clarifying the aetiology of alder decline, pointing out the key role of *Phytophthora plurivora* in this pathosystem (Bregant *et al*., 2020, 2023b; Corcobado *et al*., 2023; Seddaiu & Linaldeddu, 2020). In the present study, *P. plurivora* was isolated at very high frequency from bleeding canker, root rot and waterways in 25 sites distributed along a wide altimetric range. The high occurrence of *P. plurivora* along water streams can explain the rapid spread of this pathogen from declining to healthy riparian ecosystems, and its detrimental effects on natural vegetation.

Stream water can act as vector also for other *Phytophthora* species including a new species in the phylogenetic clade 6 described here as *Phytophthora heteromorpha* sp. nov. A multilocus sequence analysis showed that *P. heteromorpha* is a distinct species residing in subclade 6b. *Phytophthora heteromorpha* differs from the sister species *Phytophthora gregata*, *P. pseudogregata* and *P. gibbosa* based on fixed polymorphisms in the ITS, Btub and *cox*1 sequences data, as well as by a range of unique morphological traits.

In the phylogenetic analysis four European isolates available in GenBank as *Phytophthora* taxon raspberry clustered together with *P. heteromorpha.* The *nomen nudum Phytophthora* taxon raspberry, was established by Brasier *et al*., (2003) to accommodate three isolates from Australia and Europe. Since then it has been used for a heterogeneous group of isolates from Europe and Australia some of which designated by Jung *et al*. (2011) as *Phytophthora gregata*. Currently, in GenBank sequences of several *Phytophthora* species are available with a *nomen nudum*. Some of these putative species have a wide geographic distribution and belong to the ITS clade 6 (i.e. *Phytophthora* taxon hungarica, *P.* taxon paludosa, *P.* taxon sulawesiensis and *P.* taxon walnut) (Brasier *et al*., 2003; Jung *et al*., 2011; Coomber *et al*., 2023). In the last ten years, various studies have allowed the name of important pathogens to be stabilized, such as *Phytophthora bilorbang*, *P. chlamydospora*, *P. emzansi*, *P. lacustris* and *P. kelmanii* (Aghighi *et al*., 2012; Nechwatal *et al*., 2013; Hansen *et al*., 2015; Crous *et al*., 2021). The formal description of all *Phytophthora* species discovered so far is a critical point also from an applicative point of view, especially for adopting adequate quarantine measures for invasive species.

Morphologically, isolates of *P. heteromorpha* are characterized by sporangia extremely variable in shape and size and by a complex microcyclic sporulation stage, including the production of secondary zoospores (diplanetism) and microsporangia. Monomorphic diplanetism and production of microsporangia has been reported in other *Phytophthora* species (Drechsler, 1930; Waterhouse, 1970; Erwin & Ribeiro, 1996), however, the ecological significance of diplanetism in the epidemiology, as well as the possible role of microsporangia in the dispersal in nature has still been poorly studied. The zoosporic phase is of crucial importance in the dispersal of *Phytophthora* spp. along waterways (Erwin & Ribeiro, 1996). Therefore, the ecological implications related to the production of secondary zoospores by *P. heteromorpha* deserve further investigation. Another morphological trait that distinguishes *P. heteromorpha* from its closely related species is the abundant production of chlamydospores.

In addition to *P. heteromorpha* several other members belonging to the phylogenetic clade 6 were isolated from both water and symptomatic plant tissues in this study. In particular, *P. gonapodyides* and *P. lacustris* were among the most abundant species found along a wide altitude gradient from lowland to alpine areas. *Phytophthora* species from clade 6 have chiefly an aquatic and saprotrophic lifestyle, however, some species can act as opportunistic or aggressive tree pathogens (Brown & Brasier, 2007; Durán *et al*. 2008; Jung & Nechwatal 2008; Linaldeddu *et al*., 2014; Jung *et al*., 2011).

Another species frequently isolated during this study is *P. pseudosyringae*, it was obtained from 84 samples collected from 15 sites distributed in mountain areas; this organism was detected from all three substrates and nine plant hosts. *Phytophthora pseudosyringae* is a soil and airborne pathogen involved in the aetiology of several emerging diseases in temperate and low-temperature habitats worldwide (Reeser *et al*., 2011; Fajardo *et al*., 2017; Hansen *et al*., 2017).

The aerial lifestyle of *P. pseudosyringae* is related to the production of caducous sporangia, able to spread as infective propagules on epigeal plant organs, producing leaf necrosis and bleeding cankers (Bregant *et al*., 2023a). Its importance as an invasive species is growing very rapidly; recently it has been reported associated with severe diseases on 25 hosts in the Italian and Slovenian mountains (Bregant *et al*., 2023a). Pathogenicity tests on grey alder confirmed the high virulence of this species.

Finally, the results of this study confirm the wide diffusion of *P. alpina, P. cactorum, P. idaei* and *P. pseudotsugae* (clade 1a) in the riparian formations of the Dolomites. Clade 1 includes many important pathogenic species especially in crops and horticulture, such as the historical pathogens *P. infestans* and *P. nicotianae* (Grünwald & Flier, 2005; Panabieres *et al*., 2016). The most common species worldwide is *P. cactorum*, an invasive and destructive pathogen with a broad host range of herbaceous and woody hosts (Chen *et al.*, 2023). While the occurrence and host range of *P. cactorum* is well documented, very little is known about the real distribution of the other closely species *P.*

alpina, P. idaei and P. pseudotsugae (Bregant *et al*., 2023a). All these species are characterized by a relatively low cardinal temperatures, a wide thermal range of growth, caducous sporangia and chlamydospores. These traits are important to survive in cold temperature habitats, previously considered inhospitable for oomycetes (Bregant *et al*., 2023a). *Phytophthora cactorum* and *P. idaei* were shown to be very aggressive on grey alder leaves.

Conclusions

Although alders are recognized as the species most affected by *Phytophthora* spp. in riparian areas, an alarming scenario from this study emerges, since many of the main species occurring in riparian areas such as *Betula pubescens*, *Nerium oleander*, *Rhododendron ferrugineum*, *Salix purpurea*, *Tamarix gallica* are threatened by *Phytophthora* species. In particular, *P. plurivora* confirmed its wide distribution on riparian vegetation in the Mediterranean and temperate areas whereas *P. pseudosyringae* was the species more diffuse species at the highest altitude on sub-alpine vegetation. *Phytophthora plurivora* has become an invasive species in both agricultural and forest ecosystems in Europe (Linaldeddu *et al*., 2023; Schoebel *et al*., 2014). Further studies are needed to clarify its pathways and develop appropriate management strategies.

Acknowledgements

This research was partially funded by grant number DOR2305524/2023 “Monitoraggio dei marciumi radicali da *Phytophthora* negli ecosistemi forestali Italiani”, by the Land Environment Resources and Health (L.E.R.H.) doctoral course (University of Padova), by Fondo di Ateneo 2020, an internal funding by the University of Sassari.

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Chapter III

Phytophthora species involved in *Alnus glutinosa* decline in Portugal

Reprinted from: Bregant C., Batista E., Hilario S., Alves A., Linaldeddu B.T. 2023. *Phytophthora* species involved in *Alnus glutinosa* decline in Portugal. *Pathogens* 12(2): 276.

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Phytophthora species involved in *Alnus glutinosa* decline in Portugal

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Abstract: Recent field surveys conducted in five common alder ecosystems in Portugal showed the occurrence of severe canopy dieback, bleeding canker and root rot symptoms indicative of *Phytophthora* infections. Isolations from symptomatic tissues, rhizosphere and water samples yielded a total of 13 *Phytophthora* species belonging to 6 phylogenetic clades including *P. lacustris* (13 isolates), *P. multivora* (10), *P. amnicola* (9), *P. chlamydospora* (6), *P. polonica* (6), *P. bilorbang* (4), *P. plurivora* (4), *P. cinnamomi* (3), *P. asparagi* (2), *P. cactorum* (2), *P. pseudocryptogea* (2), *P. gonapodyides* (1) and *P. rosacearum* (1). Results of the pathogenicity test confirmed the complex aetiology of common alder decline and the additional risk posed by *Phytophthora multivora* to the riparian habitats in Portugal. At the same time the diversity of *Phytophthora* assemblages detected among the investigated sites suggest that different species could contribute to cause the same symptoms on this host. Two species, *P. amnicola* and *P. rosacearum* are reported here for the first time in natural ecosystems in Europe.

Keywords: emerging diseases; invasive pathogens; pathogenicity.

Citation: Bregant, C.; Batista, E.; Hilário, S.; Linaldeddu, B.T.; Alves, A. *Phytophthora* species involved in *Alnus glutinosa* decline in Portugal. *Pathogens* **2023**, *12*, 276. https://doi.org/10.3390/f14081515

Academic Editor: Paolo Gonthier

Received: 16 January 2023
Revised: 2 February 2023
Accepted: 6 February 2023
Published: 8 February 2023

1. Introduction

Alders represent an important component of European riparian and wetland vegetation. In Europe four alder species grow spontaneously, mainly along rivers, streams and damp environments, often with a pioneer behaviour fundamental for ecological successions [**1**,**2**]. In Portugal, the only species occurring naturally is common alder (*Alnus glutinosa* (L.) Gaertn.). This species is widespread in northern and central parts of the country mainly in the flooded plains and swamps at lower altitudes than the mountain riparian systems often associated with other broadleaved tree species such as *Quercus* spp. and *Salix* spp. [**3**].

Since the early 1990s alder ecosystems have been severally impacted by an emerging disease that contributed to their decline and regression in all the European continent and some areas of North America [**4**,**5**]. Typical symptoms

include a general or progressive canopy dieback, stem bleeding cankers, necrotic bark lesions at the collar and root rot [**6**].

Many studies have investigated the causes of this disease, identifying the causal agents as some members of the *Phytophthora* genus [**4**,**7**,**8**]. The disease appears to have an extremely complex aetiology; independent surveys have ascertained the occurrence of over 30 species of *Phytophthora* in declining alder ecosystems between North America and Europe [**5**, **7**, **9**,**10**]. Many of these species belong to the *Phytophthora* clade 6 *sensu* [**11**]. This clade includes organisms closely related to aquatic environments. The ecology of several *taxa* is still unclear, some species are known to have a saprotrophic or opportunistic lifestyle, while a few are reported to be aggressive pathogens [**12**,**13**].

Among the other *Phytophthora* species associated with declining alder trees, several belong to clade 2. *Phytophthora plurivora* is one of the most widespread species in declining alder ecosystems in Europe, and its pathogenicity has been confirmed using different inoculation techniques [**8**,**14**,**15**]. Whereas, in North America *P. siskiyouensis* is reported as one of the most aggressive pathogens of *Alnus* species [**16**,**17**].

Despite the several studies that have been conducted in Europe during the last three decades, many issues about the aetiology of alder decline remain to be clarified, as well as the distribution and impact of the different *Phytophthora* species among the countries. In Portugal, until now, only one study has investigated the role of *Phytophthora* species in alder decline [**18**]. The study was conducted in two alder stands along two rivers in Central Portugal confirming the involvement of two species, *Phytophthora ×alni* and *P. lacustris*, in the disease aetiology.

Therefore, given the still limited information about occurrence and impact of *Phytophthora* species in Portuguese riparian habitats and the recent discovery in Central Portugal of five riparian ecosystems with a high mortality rates of common alder trees, a study was conducted to establish the causal agents and obtain new data about the diversity and impact of *Phytophthora* species.

2. Materials and Methods

2.1. Field Surveys and Sampling procedure

Monitoring activities were conducted during spring 2022 in five natural *Alnus glutinosa* stands located in the central part of Portugal, Districts of Aveiro and Guarda, (Tab. 1). Altitude of survey sites ranged from 9 to 750 m. a.s.l.

At each site, alders were visually checked for the presence of typical *Phytophthora* disease symptoms, including wilting of foliage, shoot and twigs dieback, sudden death, bleeding cankers, root, and collar rot. In sites 2 and 3 four linear transects of 50 m were randomly established to evaluate the disease incidence and mortality rate, expressed as number of symptomatic trees out of the total number of trees (DI = n/N × 100) and number of dead trees out of the total number of trees (M = d/N × 100), respectively [**19**].

At each site, representative trees were randomly chosen for sampling (Table 1). Rhizosphere soil samples (about 1L of soil and fine roots) were collected around the collar of 38 declining alder trees. Among these, eight trees were chosen to collect bark tissues samples taking small fragments from the border of bleeding cankers on the stem. In sites 2 and 3, the occurrence of *Phytophthora* species was also monitored in the water streams using nylon mesh bags containing 10 young cork oak (*Quercus suber* L.) leaves as baits [**10**,**20**]. Nylon mesh bags were positioned near the root systems of the selected alder trees.

Table 1. Study sites information and number of stem (S), rhizosphere (R) and leaf (L) samples used for *Phytophthora* isolation.

Survey sites	Elevation (m a.s.l.)	Geographic coordinates		Number of samples
1	9	40.7035888	-8.6052296	R(4)
2	11	40.7206700	-8.5652620	R(20), S(5), L(10)
3	11	40.7141470	-8.5738595	R(10), S(3), L(10)
4	417	40.6128810	-7.5174910	R(2)
5	750	40.4106660	-7.4713180	R(2)

2.2. Isolation and Identification of Phytophthora Species

In the laboratory, samples were processed to isolate the pathogens in pure culture. Rhizosphere samples were placed in plastic boxes and flooded with 2 L of distilled water. After 24 h, pittosporum (*Pittosporum* sp.) leaves were placed on the water surface and used as baits. Boxes were kept at 18–20 °C under natural daylight and after 3–5 days, leaves showing dark spots were cut in small pieces (5 mm^2) and placed on Petri dishes containing the selective medium PDA+ [**21**].

Isolation of *Phytophthora* species was also performed directly from the necrotic tissues, taking small inner bark fragments along the border of the bleeding cankers with a sterile scalpel in aseptic conditions, and placing them in Petri dishes containing PDA+.

After ten days, the mesh bags, floating on the water surface, were collected from the stream, and transferred to the laboratory. Leaves showing necrotic dark spots were cleaned in sterile distilled water for 10 seconds, dried on sterile papers, cut in small fragments, and used for isolation of *Phytophthora* as illustrated above.

The isolates in pure culture were initially grouped in morphotypes and identified based on the colony appearance after 7 days on potato dextrose agar (PDA) and carrot agar (CA) at 20 °C in the dark, presence/absence of chlamydospores and hyphal swelling, biometric data of sporangia produced on CA plugs floating in unsterile water in petri dishes and breeding system as reported by Bregant *et al*. [**10**]. All isolates were preserved in glycerol at -80 °C, at the Department of Biology, University of Aveiro, Portugal, whereas some representative isolates of each species were stored on PDA and CA slants under oil in the culture collection of the Dipartimento Territorio e Sistemi Agro-Forestali, Università degli Studi di Padova, Italy.

2.3. Identification of Isolates

Identity of all isolates was confirmed by the analyses of DNA sequences. The genomic DNA of isolates was extracted from the mycelium of 5-day-old cultures grown on PDA at 20 °C according to the protocol reported by Möller [**22**]. The rDNA internal transcribed spacer region (ITS) was the locus chosen to be sequenced to identify isolates. The primers ITS5 and ITS4 were used to amplify and sequence the entire ITS region including the complete 5.8S gene [**23**]. Polymerase chain reactions (PCR) were performed in a final volume of 25 mL reaction mixtures containing 15.75 µL of molecular pure water, 6.25 µL of NZYTaq 2× green Master Mix (NzytechTM, Lisbon, Portugal), 1 µL of each primer at 10 pmol/µL and 1 µL of DNA template. PCR amplification conditions were performed as described by Linaldeddu *et al.* [**24**] in a Bio-Rad C1000 touch thermal cycler (USA). The nucleotide sequences were read and edited with

FinchTV 1.4.0 and then compared with reference sequences (ex-type culture or representative strains) retrieved in GenBank using the BLAST search function [**25**]. Isolates were assigned to a species when their sequences were identical (100%) to the sequence of type material or representative isolates (Table 2). Sequences from representative isolates of each species were deposited at GenBank (Table 2).

2.4 Pathogenicity Test

To confirm Koch's postulates for new host-pathogen associations, the pathogenicity of five *Phytophthora* species was tested inoculating on 1-year-old common alder seedlings grown in plastic pots (5 cm diameter, 0.5 L volume). Ten seedlings were inoculated with a representative isolate of each species, and ten were used as control. The seedlings were inoculated by wounding at the base of the stem using the protocol reported by Bregant *et al.* [**10**].

All inoculated seedlings were kept in controlled conditions at 21 °C and watered regularly for 30 days. At the end of the experimental period, seedlings were checked for the presence of internal and external disease symptoms. For each seedling the outer bark was carefully removed with a scalpel and the length of necrotic lesion surrounding each inoculation point was measured.

Re-isolation of isolates was attempted by transferring 5 pieces of inner bark taken around the margin of the necrotic lesions onto PDA+. Growing colonies were sub-cultured onto CA and PDA, incubated in the dark at 20 °C and identified by morphological and molecular analysis.

2.5 Data analysis

Pathogenicity assay data were checked for normality, then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using Fisher's least significant differences multiple range test ($p = 0.05$) after one-way ANOVA using XLSTAT 2008 software (Addinsoft).

3. Results

3.1. Symptomatology

Field surveys conducted in five common alder ecosystems in Portugal showed the widespread occurrence of severe *Phytophthora* disease symptoms on young and old alder trees. Severe disease symptoms were observed mainly in the periodically flooded areas. Disease incidence calculated in sites 2 and 3 ranged from 75 to 83%, with an average mortality rate of 32%.

Declining trees were characterized by a complex symptomatology, including extensive bleeding cankers on the lower part of the stem and sometimes on the branches with an irregular-shaped, inner bark reddish-brown necrosis as the result of death of the bark tissues (Fig. 1). In addition, different canopy symptoms such as rusty shrivelled leaves, small-size leaves, shoot blight and epicormic shoots were observed. In the late stage of the disease, infection causes a progressive or sudden decline of the whole canopy.

Figure 1. Overview of *Phytophthora* related-diseases on *Alnus glutinosa*: panoramic view that highlights a high mortality rate (**a**); alders with initial declining symptoms along a stream (**b**), severe branch dieback symptoms (**c**); bleeding cankers on stem with a wilted shoot (**d**), bleeding cankers (**e**,**f**) and root rot (**g**). On the left starting from the top, colony morphology of: *Phytophthora amnicola*, *P. asparagi*, *P. bilorbang*, *P. cactorum*, *P. chlamydospora*, *P. cinnamomi*, *P. gonapodyides*, *P. lacustris*, *P. multivora*, *P. plurivora*, *P. polonica*, *P. pseudocryptogea* and *P. rosacearum* after 7 days growth at 20 °C on CA in the dark.

3.2. Aetiology

A total of sixty-three *Phytophthora* isolates were obtained from 60 out of 66 processed samples (positivity 90.1%). Among these, 4 isolates were obtained from bleeding cankers, 36 from rhizosphere (fine roots) and 23 from leaves used as baits along the streams. Based on morphology, colony appearance and DNA sequence data 13 *Phytophthora* species namely, *P. lacustris* (13 isolates), *P. multivora* (10), *P. amnicola* (9), *P. chlamydospora* (6), *P. polonica* (6), *P. bilorbang* (4), *P. plurivora* (4), *P. cinnamomi* (3), *P. asparagi* (2), *P. cactorum* (2), *P. pseudocryptogea* (2), *P. gonapodyides* (1) and *P. rosacearum* (1) were identified (Table 2).

Table 2. *Phytophthora* isolates obtained from stem (**S**), rhizosphere (**R**) and water (**W**) samples in the investigated sites.

Species	Accession number	ITS clade	Number of samples			Sites
			Stem	Rhizosphere	Water	
P. amnicola	OQ202216	6	-	6	3	2,3
P. asparagi	OQ202217	6	-	2	-	3
P. bilorbang	OQ202218	6	-	-	4	2
P. cactorum	OQ202219	1	-	2	-	2
P. chlamydospora	OQ202220	6	-	4	2	2,3,4
P. cinnamomi	OQ202221	7	-	3	-	2
P. gonapodyides	OQ202222	6	-	1	-	3
P. lacustris	OQ202223	6	-	4	9	2,3

P. multivora	OQ202224	2	3	6	1	2,3
P. plurivora	OQ202225	2	1	3	-	1,2,4
P. polonica	OQ202226	9	-	2	4	2,3
P. pseudocryptogea	OQ202227	8	-	2	-	3,5
P. rosacearum	OQ202228	6	-	1	-	2

The most common *Phytophthora* species isolated in this study was *P. lacustris*, this species was obtained mainly from water streams. *Phytophthora amnicola* and *P. multivora* were the dominant species in rhizosphere samples, whereas *P. multivora* was the only species occurring in all types of samples (bark tissue, rhizosphere and water). *Phytophthora chlamydospora* and *P. plurivora* were the most widespread species occurring in three sites (Table 2). Seven out of 13 species isolated belong to the *Phytophthora* ITS clade 6, the most represented, followed by clade 2.

3.3. Pathogenicity

At the end of the experimental period, *Phytophthora* inoculated alder seedlings showed severe wilted symptoms associated with dark brown inner bark lesions that spread up and down from the inoculation point (Figure 2). All inoculated *Phytophthora* species proved to be pathogenic on common alder

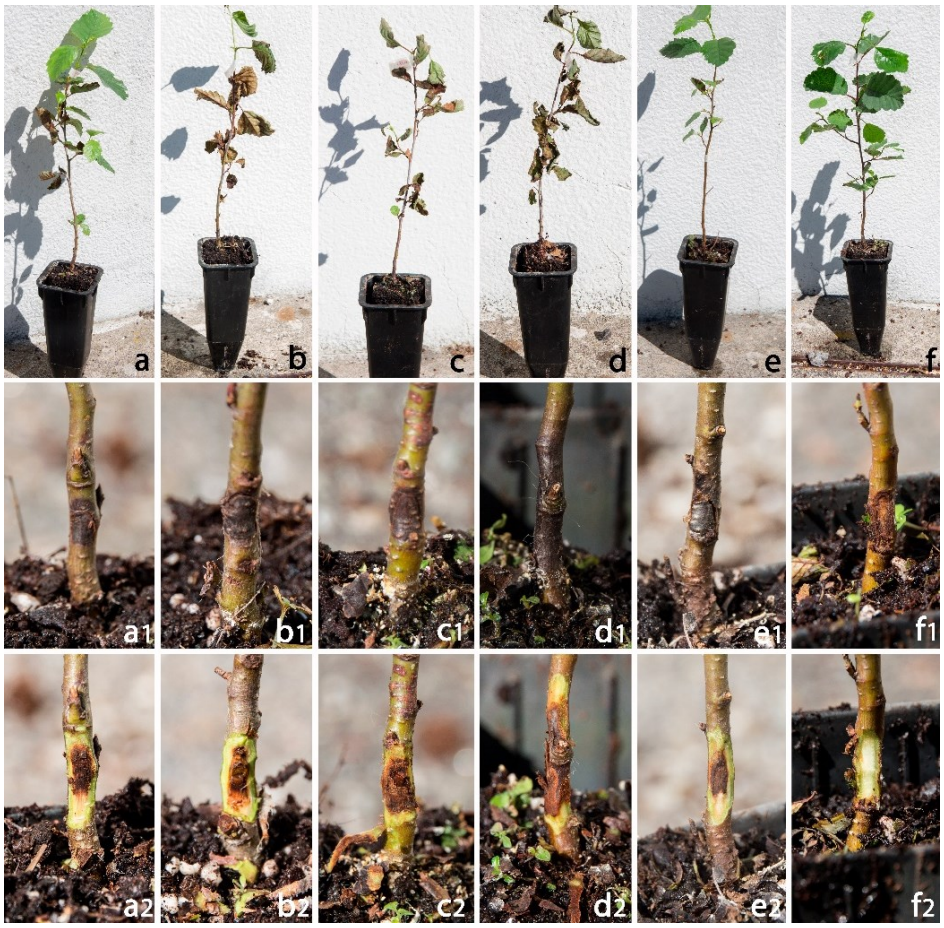

Figure 2. Symptoms observed on common alder seedlings after 30 days from inoculation with *Phytophthora amnicola* (**a-a2**), *P. asparagi* (**b-b2**), *P. chlamydospora* (**c-c2**), *P. multivora* (**d-d2**) and *P. rosacearum* (**e-e2**). Control seedlings (**f-f2**).

The average lesion length differed significantly among species (Table 3). The lesions caused by *P. multivora* were significantly larger than those caused by the other species. Necrotic inner bark lesions caused by *P. multivora*, *P. chlamydospora*, *P. asparagi* and *P. amnicola* progressively girdled the stem causing wilting symptoms and sudden death of the seedlings (Tab. 3). Control plants inoculated with sterile PDA plugs remained symptomless. All inoculated species were successfully re-isolated (100%) from the margin on necrotic inner bark lesions of all seedlings. No *Phytophthora* isolates or other microorganisms were re-isolated from control seedlings.

Table 3. Mean lesion length ± standard deviation caused by each *Phytophthora* species on common alder seedlings.

Species	Isolates	Mean Lesion Length (mm)*	Exudates	Wilting	Re-isolation (%)
P. amnicola	CBP28	11.0 ± 4.8bc	-	30%	100
P. asparagi	CBP23	12.2 ± 4.7bc	-	40%	100
P. chlamydospora	CBP16	15.5 ± 4.8b	-	30%	100
P. multivora	CBP56	41.2 ± 14.7a	40%	80%	100
P. rosacearum	CBP81	8.5 ± 3.1cd	-	-	100
Control	-	2.5 ± 1.4d	-	-	-
Critical value	-	2.006			

* Values in column with the same letter do not differ significantly at p = 0.05, according to LSD multiple range test.

4. Discussion

The extensive survey of *Phytophthora* related diseases conducted in Central Portugal showed the occurrence of 13 species, *P. amnicola*, *P. asparagi*, *P. bilorbang*, *P. cactorum*, *P. chlamydospora*, *P. cinnamomi*, *P. gonapodyides*, *P. lacustris*, *P. multivora*, *P. plurivora*, *P. polonica*, *P. pseudocryptogea* and *P. rosacearum* in five riparian habitats (rivers and streams) characterized by a high common alder mortality. Ten out of 13 species were recovered from natural declining common alder ecosystems in previous studies in Europe [**7**,**8**,**9**,**10**,**26**,**27**].

Most species found in this study are classified in the ITS Clade 6 *sensu* [**11**,**28**]. This major clade includes a large number of saprotrophic or weak opportunistic pathogens strongly linked to aquatic environments [**12**]; therefore, it is not surprising that *P. lacustris* was found to be the dominant species. Its presence in Portugal associated to declining alder formations was recently documented by Kanoun-Boulè [**18**]. Three of the other species belonging to clade 6, *P. bilorbang*, *P. chlamydospora* and *P. gonapodyides* are very common in temperate riparian habitats of Europe and other continents, often in association with declining alders [**9**,**10**,**29**] whereas *P. amnicola, P. asparagi* and *P. rosacearum* are reported here for the first time on declining common alder.

Phytophthora amnicola was originally described in 2012 in Western Australia [**30**]. Its lifestyle appears strongly related to water, this is corroborated by the data obtained in this survey, but little is known regarding the ecology and potential impact of this species in Portugal.

From the rhizosphere of two alder trees colonies of *P. asparagi* were obtained. This species was described in the USA in 2012, but it had been known for a long time and the name is currently considered invalid [**31**,**32**]. *Phytophthora asparagi* has been reported in different countries as a pathogen on ornamental and crop plants [**31**,**33**]. Some recent studies have demonstrated that this species is widespread in Mediterranean environments on several species of the Mediterranean maquis [**34**,**35**]. Its diffusion in natural areas appears closely

linked to the white asparagus (*Asparagus albus*), a preferential host that can facilitate host jumps [**34**].

Phytophthora rosacearum was isolated for the first time in California on *Malus* sp. and later from other diseased crops, such as pear in California and pomegranate in Turkey [**36**,**37**,**38**] The recovery of *P. rosacearum* on common alder in Portugal represents the first report of this species in a natural habitat and in Europe. Based on the results obtained in the pathogenicity test *P. rosacearum* can be considered a weak pathogen of common alder compared to other *Phytophthora* species.

The second most represented clade consists of two species, *P. multivora* and *P. plurivora*. *Phytophthora multivora* was the only species found in all types of samples monitored (stem bleeding cankers, necrotic fine roots and baited leaves along stream). The discovery of this polyphagous pathogen causing root rot and mortality of common alder in Portugal poses an additional threat to alder stands in Europe. *Phytophthora multivora* was previously reported causing root and collar rot lesions on *Agathis australis*, *Agonis flexuosa*, *Banksia* spp., *Corymbia calophylla*, *Eucalyptus* spp., *Rubus anglocandicans*, *Wollemia nobilis* in Australia and New Zealand and on *Acacia mearnsii*, *Alnus glutinosa*, *Araucaria araucana*, *Quercus* spp., *Rhododendron* sp., *Salix fragilis* in natural areas in Chile, Czech Republic, Germany, Hungary, New Zealand and South Africa [**39**,**40**,**41**,**42**,**43**,**44**,**45**,**46**,**47**,**48**]. The underbark inoculation test confirmed the aggressiveness of this emerging pathogen on common alder. Previous studies have ascertained its pathogenicity on *Agathis australis*, *Agonis flexuosa*, *Corymbia* spp., *Eucalyptus* spp., *Banksia* spp., *Rubus anglocandicans* and *Wollemia nobilis* [**40**,**42**,**44**,**45**,**49**,**50**].

The other species consistently detected from bark lesions and rhizosphere was *P. plurivora*, a plurivorous pathogen involved in the aetiology of several diseases of woody hosts in six continents [**51**,**52**,**53**,**54**]. The high isolation frequency of *P. plurivora* in this study is in accordance with results of previous studies conducted on declining common alder trees in Italy [**8**,**10**].

Finally, the other four species were recovered less frequently. Among these *P. cactorum, P. polonica* and *P. pseudocryptogea* are detected for the first time related to declining forest in Portugal but already known on common alder in other European countries [**10**,**55**]. Whereas the discovery of *P. cinnamomi* in riparian habitats was of particular concern due to its wide host range and the impact of this pathogen on oak forests in Portugal [**56**].

The fulfilment of Koch postulates for the five species tested in this study expands the list of pathogenic *Phytophthora* species on common alder to 29 [table S1], suggesting that the disease may be caused by more than one pathogen under different environmental conditions.

5. Conclusions

While *P. ×alni* has been getting most attention during the last decades, the most common species isolated from declining alder trees in Europe is *P. plurivora* [**9**,**10**,**27**]. At the same time, the current trend in discovering an increasing number of pathogenic species from declining alder trees emphasizes how much more we need to learn about *Phytophthora* biodiversity and impact in riparian ecosystems. More in general this work contributed to expanding knowledge on the biodiversity of *Phytophthora* species in natural areas of Portugal with eight new reports (Table S2).

Author Contributions: Conceptualization: C.B., BT.L. and A.A.; methodology, C.B., B.T.L. and A.A.; formal analysis, C.B., E.B. and S.H.; investigation, C.B., E.B. and S.H.;

resources, A.A and B.T.L.; writing—original draft preparation, C.B.; writing—review and editing, B.T.L. and A.A.; supervision, B.T.L. and A.A.; funding acquisition, A.A. and B.T.L. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: This research has been financially supported by the Land Environment Resources and Health (L.E.R.H.) doctoral course (University of Padova). Thanks are due to the Portuguese Foundation for Science and Technology (FCT/MCTES) for the financial support to CESAM (UIDP/50017/2020 + UIDB/50017/2020 + LA/P/0094/2020), and the PhD grants of Sandra Hilário (SFRH/BD/137394/2018) and Eduardo Batista (PD/BD/135535/2018).

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary materials

Table S1. *Phytophthora* species reported in natural ecosystems in Portugal.

Species	Host	References
P. alticola	*Eucalyptus globulus*	[**57**]
P. amnicola	*Alnus glutinosa*	This study
P. asparagi	*A. glutinosa*	This study
P. bilorbang	water	This study
P. cactorum	*A. glutinosa*	This study
P. cambivora	*Castanea sativa*	[**58,59**]
P. castanetorum	*C. sativa*	[**60**]
P. chlamydospora	*A. glutinosa*	This study
P. cinnamomi	*A. glutinosa, Arbutus unedo, Calluna vulgaris, Castanea sativa, Cistus crispus, C. ladanifer, C. populifolius, C. salvifolius, E. globulus, Genista triacanthos, Pinus pinaster, Quercus rotundifolia, Q. robur, Q. suber, Ulex* spp.	[**56,57,61,62,63,64**]; this study
P. condilina	water	[**65**]
P. gonapodyides	*A. glutinosa*, water	[**65**]; this study
P. inundata	water	[**65**]
P. lacustris	*A. glutinosa*	[**18**]
P. multivora	*A. glutinosa*	This study
P. plurivora	*A. glutinosa*, water	[**65**], this study
P. polonica	*A. glutinosa*	This study
P. pseudocryptogea	*A. glutinosa*, water	[**65**], this study
P. psychrophila	*Q. rotundifolia*	[**66**]
P. quercina	*Q. rotundifolia, Q. pyrenaica*	[**60**]
P. rosacearum	*A. glutinosa*	This study
P. ramorum	*Viburnum* sp.	[**67**]
P. ×alni	*A. glutinosa*	[**18**]

Table S2. *Phytophthora* species reported as pathogenic on *Alnus glutinosa*.

Species	References
P. amnicola	This study
P. asparagi	This study
P. acerina	[**8**]
P. cactorum	[**7**]
P. cambivora	[**68**,**69**]
P. chlamydospora	This study
P. cinnamomi	[**68**]
P. citrophthora	[**70**]
P. cryptogea	[**68**]
P. gallica	[**71**]
P. gonapodyides	[**7**]
P. inundata	[**72**]
P. lacustris	[**7**,**72**]
P. megasperma	[**7**]
P. multivora	This study
P. nicotianae	[**70**]
P. palmivora	[**70**]
P. plurivora	[**7**,**8**,**15**,**73**,**74**]
P. polonica	[**55**]
P. pseudocryptogea	[**8**]
P. pseudosyringae	[**75**]
P. ramorum	[**73**]
P. rubi	[**68**]
P. syringae	[**55**]
P. rosacearum	This study
P. uniformis	[**15**,**68**]
P. ×alni	[**15**,**68**]
P. ×multiformis	[**15**,**68**]
Phytophthora taxon raspberry	[**72**]

Chapter IV

Phytophthora mediterranea sp. nov, a new species closely related to *Phytophthora cinnamomi* from nursery plants of *Myrtus communis* in Italy

Reprinted from: Bregant C., Mulas A.A., Rossetto G., Deidda A., Maddau L., Piras G., Linaldeddu B.T. 2021. *Phytophthora mediterranea* sp. nov, a new species closely related to *Phytophthora cinnamomi* from nursery plants of *Myrtus communis* in Italy. *Forests* 12(6): 682.

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Phytophthora mediterranea sp. nov., a New Species Closely Related to *Phytophthora cinnamomi* from Nursery Plants of *Myrtus communis* in Italy

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Citation: Bregant, C.; Mulas, A.A.; Rossetto, G.; Deidda, A.; Maddau, L.; Piras, G.; Linaldeddu, B.T. *Phytophthora mediterranea* sp. nov., a New Species Closely Related to *Phytophthora cinnamomi* from Nursery Plants of *Myrtus communis* in Italy. *Forests* **2021**, *12*, 672. https://doi.org/10.3390/f12060682

Academic Editor: Paulo A. Zaini

Received: 04 May 2021
Accepted: 25 May 2021
Published: 27 May 2021

Abstract: Monitoring surveys of *Phytophthora* related diseases in four forest nurseries in Italy revealed the occurrence of fourteen *Phytophthora* species to be associated with collar and root rot on fourteen plants typical of Mediterranean and alpine regions. In addition, a multilocus phylogeny analysis based on nuclear ITS and ß-tubulin and mitochondrial *cox*1 sequences, as well as micromorphological features, supported the description of a new species belonging to the phylogenetic clade 7c, *Phytophthora mediterranea* sp. nov. *Phytophthora mediterranea* was shown to be associated with collar and root rot symptoms on myrtle seedlings. Phylogenetically, *P. mediterranea* is closely related to *P. cinnamomi* but the two species differ in 87 nucleotides in the three studied DNA regions. Morphologically *P. mediterranea* can be easily distinguished from *P. cinnamomi* on the basis of its smaller sporangia, colony growth pattern and higher *optimum* and *maximum* temperature values. Data from the pathogenicity test showed that *P. mediterranea* has the potential to threaten the native Mediterranean maquis vegetation. Finally, the discovery of *P. cinnamomi* in alpine nurseries, confirms the progressive expansion of this species towards cold environments, probably driven by climate change.

Keywords: cryptic species; emerging diseases; global trade; biosecurity

1. Introduction

Over the last few years, the horticultural industry has had a progressive expansion worldwide, which resulted in an increase in the trade of plants and commodities among continents [**1**,**2**]. In Italy, the horticulture industry is an important component of the agricultural sector; its production covers over 30,000 hectares with 100,000 employees and has a sellable production of about 2.5 billion euros per year [**3**].

Over the last few decades, the exponential increase in plant materials and commodities traded, together with limited and often ineffective diagnostic

measures to detect pests and pathogens at the borders have contributed to a rapid diffusion of exotic and invasive species worldwide [**4**,**5**]. Among the most destructive pathogens that are introduced annually through trade and transport of plant material, many belong to the genus *Phytophthora* [**6**,**7**].

The genus *Phytophthora* encompasses a range of morphologically and ecologically diverse *taxa* grouped into 12 well-defined phylogenetic clades [**8–10**]. Some species have a saprophytic or opportunistic lifestyle, whereas others are aggressive pathogens that cause root and collar rot, bleeding canker and leaf blight symptoms in a huge number of plant species [**11**,**12**]. In particular, *Phytophthora* spp. are responsible for severe outbreaks with reduced vitality and quality of nursery plants [**13**]. In addition to the direct economic losses caused to the horticulture industry, *Phytophthora* spp. pose a threat to biodiversity of natural ecosystems as they are often introduced through the seedlings used for reforestation and restoration programmes [**14–16**]. In this regard, the *P. ramorum* and *P. lateralis* outbreaks in North America and *P. cinnamomi* in Australia are emblematic, having caused the devastation of extensive ecosystems [**17–20**].

Due to intensive cultivation techniques and the occurrence of different plant species in an often-limited space, nurseries are an ideal place for many *Phytophthora* species [**13**]. Moreover, the proximity of many plant species and possible encounters between genetically related species can easily give rise to new host–pathogen associations as well as hybridization phenomena [**21**]. High-humidity conditions and recurrent water irrigation can also favour the reproduction and dissemination of these pathogens [**22–25**]. Many *Phytophthora* species, in fact, need water to complete their biological cycle [13,26]. Furthermore, many agronomic practices such as recycling of soil and pots could be pervasive to increase *Phytophthora* inoculum inside nurseries [**13**,**27–29**]. At the same time, the intensive use of chemicals can promote the formation of fungicide-resistance in pathogen populations [**30–32**].

Phytophthora occurrence in North American, Australian and European nurseries is well documented [**33–39**]. The increased attention paid to *Phytophthora* species in the last decade has allowed the knowledge about biology and ecology of many species to be expanded, as well as the discovery of about 30 new species in nurseries [**10**]. Some of these new *taxa* are related to the aquatic environment and characterized by a saprophytic lifestyle [**40–44**]. In contrast, others such as *P. niederhauserii* and *P. kernoviae* are polyphagous and potentially invasive [**45**,**46**].

With the expansion of ornamental horticulture, *Phytophthora* related diseases are also becoming a serious problem in Italy. Recent studies have revealed a very high diversity of *Phytophthora* species in ornamental nurseries [**47**,**48**]. This is linked both to the widespread presence of invasive and ubiquitous species such as *P. nicotianae* and *P. palmivora*, but also of new species, such as *P. parvispora*, a cryptic species closely related to *P. cinnamomi* [**48**,**49**]. At the same time, the discovery of rare species on nursery plants, such as *Phytophthora pistaciae*, characterized by a limited geographic distribution points out the risks posed by nursery material to the conservation and integrity of natural ecosystems in the new areas [**50**].

Therefore, given the constant discovery of new or rare *Phytophthora* species in Italian nurseries, a thorough study was conducted to isolate, identify and characterize the main *Phytophthora* species associated with symptomatic plants in four nurseries spanning from the Mediterranean to the alpine climate region.

2. Materials and Methods

2.1. Surveys and Sampling Procedure

Field surveys were conducted from spring 2019 to autumn 2020 in four Italian forest nurseries located in Sardinia (N1 and N2), Veneto (N3) and Friuli Venezia Giulia (N4) regions (Table 1). In each nursery, all potting plants between 6 months and 5 years were visually checked for the presence of *Phytophthora* related disease symptoms such as chlorosis, defoliation, shoot blight, sudden death, as well as collar and root rot. Among the monitored plants, 76 were randomly selected for diagnostic analyses. The sampled species included *Abies alba*, *Arbutus unedo*, *Alnus incana*, *Castanea sativa*, *Fagus sylvatica*, *Helichrysum italicum*, *Ilex aquifolium*, *Juglans regia*, *Laurus nobilis*, *Lavandula officinalis*, *Myrtus communis*, *Phyllirea latifolia*, *Pistacia lentiscus* and *Quercus ilex* (Table 1). The plant samples were sealed in plastic bags, labelled and used for *Phytophthora* isolations within 24-48h.

Table 1. Nurseries information and plant species monitored for *Phytophthora*.

Nursery	Elevation (m. a.s.l.)	Geographic Coordinates		Plant Species *
N1	860	40°25′54″N	8°58′43″E	Cs (12), Ia (6), Jr (12), Ln (6), Phl (6)
N2	16	39°57′43″N	8°36′02″E	Au (4), Hi (4), Lo (2), Mc (3), Pl (7), Qi (6)
N3	1077	46°06′54″N	12°26′05″E	Aa (2), Fs (2)
N4	191	46°11′51″N	13°14′13″E	Ai (4)

* In brackets the number of plants collected: *Abies alba* (Aa), *Arbutus unedo* (Au), *Alnus incana* (Ai), *Castanea sativa* (Cs), *Fagus sylvatica* (Fs), *Helichrysum italicum* (Hi), *Ilex aquifolium* (Ia), *Juglans regia* (Jr), *Laurus nobilis* (Ln), *Lavandula officinalis* (Lo), *Myrtus communis* (Mc), *Phyllirea latifolia* (Phl), *Pistacia lentiscus* (Pl) and *Quercus ilex* (Qi).

2.2. Isolation of Phytophthora Species

In the laboratory, plant samples were initially checked for collar and root symptoms and then used for *Phytophthora* isolation. From each sample about 300g of rhizosphere soil and roots were positioned inside plastic containers with 2 L of distilled water. After 24 h the water surface was cleaned and young *Quercus suber* and *Sambucus nigra* leaves placed on the surface as bait [**51**]. Containers were kept at 20 °C under natural daylight for 3-7 days. Leaves showing necrotic spots were cut in small pieces (5 mm^2) and placed on petri dishes containing potato dextrose agar (PDA 39 g/L, Oxoid Ltd., Basingstoke, UK) supplemented with 100 mL/L of carrot juice, 0.013 g/L of pimaricin and 0.05 g/L of hymexazol (PDA+) [**50**]. Hyphal tips typical of *Phytophthora* from the emerging colonies were sub-cultured on PDA and carrot agar (CA) [**11**] and incubated at 20 °C in the dark.

Isolations of *Phytophthora* species were also performed from collar and root lesions. After removing the outer bark, inner bark fragments were aseptically cut from the margin of necrotic lesions with a sterile scalpel and placed onto 90 mm Petri dishes containing PDA+. The dishes were incubated in the dark at 20 °C and examined every 6–12 h. Pure colonies were obtained as described above.

2.3. Morphological Identification and Characterization of Isolates

All *Phytophthora* isolates were grouped into morphotypes on the basis of colony growth patterns including surface and reverse colony appearance observed after 7 days of incubation on PDA and CA at 20 °C in the dark and morpho-biometric data of oogonia and sporangia. To enhance sporangia

production, CA plugs (5 mm diameter) of each isolate, taken from 4-day-old colonies, were positioned inside petri dishes containing unsterile pond water and three cork oak fine roots (1 cm long). Petri dishes were kept at 20 °C in the dark and sporangia production was assessed every 12 h for 4 days.

Colony growth patterns of the new species were evaluated on 7-day-old cultures incubated at 25 °C in the dark on CA, PDA and malt extract agar (MEA, 20 g/L, Oxoid Ltd.). In addition, size and shape of fifty chlamydospores, hyphal swellings and sporangia were recorded. Cardinal temperatures for growth were evaluated on 90 mm CA plates incubated at 5, 10, 15, 20, 25, 30, 32, 35, 37 and 40 °C (±0.5 °C) in the dark for 96 h. Five replicate plates for each isolate were made. An isolate of both *P. parvispora* (CB86) and *P. cinnamomi* (CB21) obtained in the study were also included in the growth bioassay for comparison.

Measurements and photos of the main morphological structures were taken at 400× and 600× magnification and recorded using the software Motic Images Plus 3.0 paired with a Moticam 10+ camera connected to a Motic BA410E microscope. Sporangia dimensions are presented as mean values ± standard deviation.

Representative isolates of each species were stored on PDA and CA slants under oil in the culture collection of the Dipartimento Territorio e Sistemi Agro-Forestali, Università degli Studi di Padova. Ex-type culture of the new species was deposited at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, and nomenclatural data in MycoBank (www.MycoBank.org). The holotype was lodged with the herbarium of Westerdijk Fungal Biodiversity Institute as a dried culture on CA.

2.4. DNA Extraction, Polymerase Chain Reaction (PCR) Amplification and Sequencing

For all isolates genomic DNA was extracted from mycelium of 5-day-old colonies grown on PDA at 20 °C using Instagene Matrix (BioRad Laboratories, Hercules, CA, USA). The entire internal transcribed spacer (ITS) region of the ribosomal DNA, including the 5.8S rRNA gene, was amplified and sequenced using primers ITS1 and ITS4 [**52**]. ITS sequences were used to confirm the identification at species level. For four isolates belonging to the clade 7 [**8**] including those of the new species, other two DNA regions namely *ß*-tubulin (Btub) and cytochrome c oxidase subunit I (*cox*1), were amplified and sequenced using the primer-pairs TUBUF2/TUBUR1 and FM84/FM83 [**53**,**54**], respectively. Polymerase chain reactions (PCR) were performed as described in Bregant *et al*. [**51**].

PCR products were purified using a EUROGOLD gel extraction kit according to the manufacturer's instructions (EuroClone S.p.A., Pero, Italy). Both strands were sequenced by BMR Genomics DNA sequencing service (www.bmr-genomics.it). Sequences were edited with FinchTV v1.4.0 (Geospiza, Inc., http://www.geospiza.com/finchtv) and compared with sequences of ex-type culture available in GenBank using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov). New sequences were deposited in GenBank (Tables 2 and 3). Alignments and trees are available in TreeBase (study ID 28180).

Table 2. *Phytophthora* isolates used in the phylogenetic analysis. Ex-type cultures are reported in bold and newly generated sequences are indicated in italics.

Species	**Code**	**Host**	**GenBank Accession Number**		
			ITS	**Btub**	***Cox*1**
P. asiatica	**CBS 133347**	*Pueraria lobata*	AB688422	AB539560	AB740169

P. attenuata	**CBS 141199**	*Castanopsis carlesii*	KU517154	KU899277	LC595899
P. cajani	**P3105**	*Cajanus cajan*	MG783386	MH493912	MH136859
P. cinnamomi	**CBS 144.22**	*Cinnamomum* sp.	MG865473	MH493920	MH136869
P. cinnamomi	CB21	*Abies alba*	*MW892397*	*MW900442*	*MW900446*
P. cambivora	**P19997**	*Castanea sativa*	MG783387	MH493913	MH136860
P. europaea	**CBS 109049**	*Quercus robur*	MG865488	MH493935	MH136884
P. flexuosa	**CBS 141201**	*Fagus hayatae*	KU517152	KU899302	LC595910
P. formosa	**CBS 141203**	*Araucaria* sp.	KU517153	KU899270	LC595912
P. fragariae	**CBS 209.46**	*Fragaria ×ananassa*	MG865494	MH493938	MH136890
P. fragariaefolia	**CBS 135747**	*Fragaria ×ananassa*	MG865495	MH493939	MH136891
P. intricata	**CBS 141211**	*Quercus tarokoensis*	KU517155	KU899284	LC595921
P. niederhauserii	**P10616**	*Hedera helix*	MG865552	MH493988	MH136944
P. melonis	**CBS 582.69**	*Cucumis sativus*	MG865536	MH493974	MH136931
P. mediterranea	**CB84**	*Myrtus communis*	*MW892398*	*MW900443*	*MW900447*
P. mediterranea	CB85	*M. communis*	*MW892399*	*MW900444*	*MW900448*
P. nagaii	**CBS 133248**	*Rosa* sp.	MG865547	MN207274	MH136940
P. parvispora	**CBS 132772**	*Arbutus unedo*	KC478667	KC609402	KC609413
P. parvispora	CB86	*A. unedo*	*MW892401*	*MW900445*	*MW900449*
P. pisi	**CBS 130350**	*Pisum sativus*	MG865567	MH494000	MH477754
P. pistaciae	**P19883**	*Pistacia vera*	KT183043	KX251749	LC595934
P. rubi	**CBS 967.95**	*Rubus idaeus*	MG865584	MH494011	MH136976
P. sojae	**CBS 382.61**	*Glycine max*	MG865587	MN207279	MH136979
P. tyrrhenica	**CBS 142301**	*Quercus ilex*	KU899188	KU899265	LC595950
P. uliginosa	**CBS 109054**	*Q. robur*	MG865597	MH494023	MH136988
P. uniformis	**P16206**	*Alnus glutinosa*	MK496514	MH493905	MH136992
P. vignae	**P3019**	*Vigna sinensis*	MG865598	MH494024	MH136989
P. vulcanica	**CBS 141216**	*Fagus sylvatica*	MF036209	MF036235	LC595951
P. ×alni	**IMI 392314**	*A. glutinosa*	MK496513	MH493903	MH136991
P. ×heterohybrida	**CBS 141207**	Water	KU517151	KU899290	LC595953
P. ×incrassata	**CBS 141209**	Water	KU517156	KU899286	LC595954
P. ×multiformis	**P16202**	*A. glutinosa*	MG783372	MH493904	MK493472

Table 3. Number of isolates of *Phytophthora* species obtained from monitored plants in the investigated nurseries.

Species	Accession Number	ITS Clade	Plant Species *	Nursery
P. acerina	MW892395	2	Ai (1)	N4
P. bilorbang	MW959911	6	Phl (2)	N1
P. cactorum	MW892396	1	Cs (1)	N1
P. cinnamomi	MW892397	7	Cs (8), Fs (2), Aa (2), Jr (8), Qi (6)	N1-2-3
P. citrophthora	MW959916	2	Ln (1)	N1
P. mediterranea	MW892398	7	Mc (2)	N2
P. megasperma	MW959913	6	Ln (3)	N1
P. nicotianae	MW892400	1	Lo (1), Pl (2), Hi (4), Mc (2)	N2
P. palmivora	MW959917	4	Phl (5)	N1
P. parvispora	MW892401	7	Au (3)	N2
P. pistaciae	MW892402	7	Pl (2)	N2
P. plurivora	MW892403	2	Ai (3)	N4
P. pseudocryptogea	MW959912	8	Ln (4)	N1

P. pseudosyringae	MW959914	3	Ia (3)	N1
P. psychrophila	MW959915	3	Ia (1)	N1

* In brackets the number of *Phytophthora* isolates on: *Abies alba* (Aa), *Arbutus unedo* (Au), *Alnus incana* (Ai), *Castanea sativa* (Cs), *Fagus sylvatica* (Fs), *Helichrysum italicum* (Hi), *Ilex aquifolium* (Ia), *Juglans regia* (Jr), *Laurus nobilis* (Ln), *Lavandula officinalis* (Lo), *Myrtus communis* (Mc), *Phyllirea latifolia* (Phl), *Pistacia lentiscus* (Pl) and *Quercus ilex* (Qi).

2.5. Phylogenetic Analysis

ITS, Btub and *cox*1 sequences of four isolates obtained in this survey were compiled in a dataset together with 78 sequences from 26 *Phytophthora* species representative of described species in the clade 7 (including ex-type culture) for which molecular data are available in GenBank (Table 2).

Sequence alignments were performed with ClustalX v. 1.83 [**55**], using the parameters reported in Bregant *et al*. [**51**]. Alignments were checked and edited with BioEdit Alignment Editor v. 7.2.5 [**56**]. Phylogenetic analyses were completed with MEGA-X 10.1.8 [**57**]. All gaps were included in the analyses. The best model of DNA sequence evolution was determined automatically by the software. Maximum likelihood (ML) analysis was performed with a neighbour-joining (NJ) starting tree generated by the software. A bootstrap analysis (1000 replicates) was used to estimate the robustness of nodes.

2.6. Pathogenicity Test

Pathogenicity of the new *Phytophthora* species was tested on 3-year-old myrtle (*Myrtus communis*) and lentisk (*Pistacia lentiscus*) seedlings grown in plastic pots (20 cm diameter, 2 L volume). Eight seedlings of each plant species were inoculated with the isolate CB84 (ex-type culture). In addition, the same number of each plant species were inoculated with the isolate CB21 of *P. cinnamomi* for comparison. Finally, the same number of seedlings were used as control. Inoculation was performed at the collar. The inoculated point was surface-disinfected with 70% ethanol and a small piece of outer bark (5 mm diameter) was removed with a flame-sterilised cork borer and replaced with an agar-mycelium plug of the same size, taken from the margin of an actively growing colony on CA. The inoculation point was covered with cotton wool soaked in sterile water and wrapped with an aluminium foil to retain moisture. Controls were inoculated with a sterile CA plug.

All inoculated seedlings were kept in a climatic chamber at 25 °C and watered every two days until the end of the experimental period. After 20 days, all plants were checked for the presence of disease symptoms and the length of necrotic lesion surrounding the inoculation site was measured after removing the outer bark with a sterile scalpel.

Re-isolation was performed by transferring 10 pieces of inner bark taken around the margin of the necrotic lesions onto PDA+. Growing colonies were sub-cultured onto CA and PDA, incubated in the dark at 20 °C and identified by morphological and molecular analysis (ITS region) to confirm Koch's postulates.

2.7. Data Analysis

Data from the pathogenicity assay were first checked for normality and then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using Fisher's least significant differences multiple range test (p = 0.05) after one-way ANOVA using XLSTAT 2008 software (Addinsoft).

3. Results

3.1. Symptomatology

Phytophthora related diseases were observed in all investigated nurseries. Regardless of the species, potted plants showed wilting foliage, chlorosis, stunted growth and sudden death symptoms (Figure 1). Sudden death was very common in plants of the alpine nursery (N3) and, in particular, of silver fir and beech seedlings.

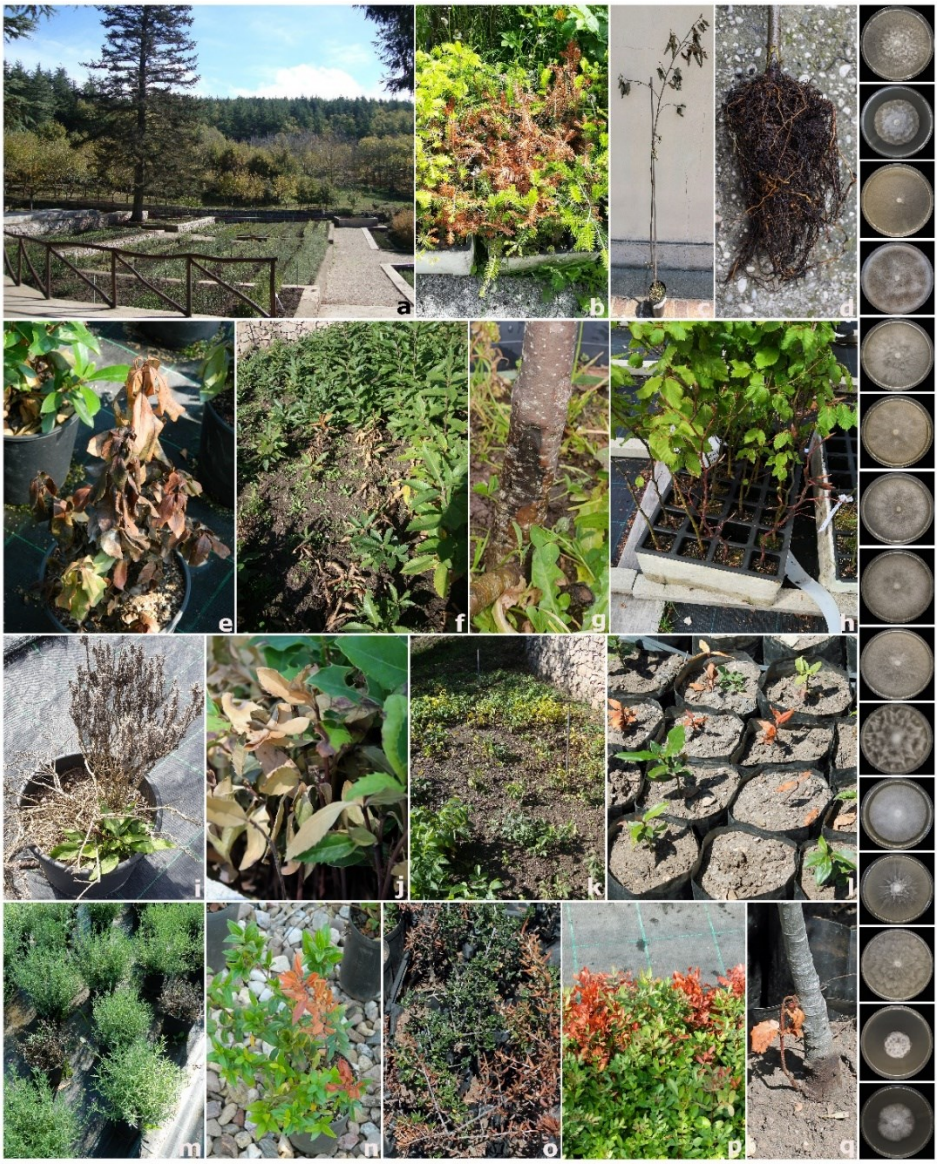

Figure 1. Overview of the forest nursery N1 (a), symptoms of wilting, sudden death and collar and root rot observed on: *Abies alba* (b), *Alnus incana* (c,d), *Arbutus unedo* (e), *Castanea sativa* (f,g), *Fagus sylvatica* (h), *Helichrysum italicum* (i), *Ilex aquifolium* (j), *Juglans regia* (k), *Laurus nobilis* (l), *Lavandula officinalis* (m), *Myrtus communis* (n), *Phyllirea latifolia* (o), *Pistacia lentiscus* (p) and *Quercus ilex* (q) seedlings. On the right from the top, colony morphology of: *Phytophthora acerina*, *P. bilorbang*, *P. cactorum*, *P. cinnamomi*, *P. citrophthora*, *P. mediterranea*, *P. megasperma*, *P. nicotianae*, *P. palmivora*, *P. parvispora*, *P. pistaciae*, *P. plurivora*, *P. pseudocryptogea*, *P. pseudosyringae* and *P. psychrophila* after 7 days growth at 25 °C on CA in the dark.

All canopy symptoms described above were associated with collar and root rot symptoms. In most cases, the root system was completely compromised and fine roots absent (Figure 1). The mortality rates, especially in one-year-old seedlings, were very high with losses close to 60–70%.

3.2. Phytophthora Species Associated with Nursery Plants

Isolations performed on 76 symptomatic plants yielded a total of 66 *Phytophthora* isolates. The largest number of isolates (36) was obtained from site N1, followed by site N2 (22), whereas a total of 8 isolates were obtained from sites N3 and N4.

On the basis of micromorphological features and ITS sequence data, fourteen *Phytophthora* species belonging to seven phylogenetic clades were identified: *P. cinnamomi* (26 isolates), *P. nicotianae* (9), *P. palmivora* (5), *P. pseudocryptogea* (4), *P. megasperma* (3), *P. plurivora* (3), *P. parvispora* (3), *P. pseudosyringae* (3), *P. pistaciae* (2), *P. bilorbang* (2), *P. acerina* (1), *P. cactorum* (1), *P. citrophthora* (1) and *P. psychrophila* (1). For each species BLAST searches against GenBank showed 100% identity to reference sequences of representative strains including those of ex-type culture (Table 3). Two isolates obtained from declining myrtle seedlings could not be assigned on the basis of morphological features and ITS sequence data to any formally described species or informally designated *Phytophthora* and were therefore considered a new *taxon* unknown to science.

Among the detected species, *P. cinnamomi* was the dominant one, being isolated from five different hosts in three nurseries (Table 3). *Phytophthora nicotianae* was the most abundant species in the nursery N2. The other species were isolated only from a single host. Interestingly, some of the monitored plant species were positive for two or three *Phytophthora* species.

3.3. Phylogenetic Analysis

Phylogenetic relationships among the *Phytophthora* species belonging to clade 7 and four representative isolates obtained in this study were elucidated using a multilocus analysis based on the sequences of ITS, Btub and *cox1* regions.

Fragments of approximately 800, 920 and 1020 bp were obtained for ITS, Btub and *cox1* regions, respectively. Individual gene phylogenies revealed similar tree topologies, indicating that the three loci could be combined (data not shown). The ML evolutionary reconstruction allowed for the differentiation of 27 distinct lineages within clade 7, corresponding to 27 species and hybrids (Figure 2). The four isolates obtained in this study were distributed into three sub-clades in clade 7c (Figure 2). In particular, isolate (CB21) clustered with ex-culture type of *P. cinnamomi* and isolate (CB86) with ex-culture type of *P. parvispora*. The remaining two isolates clustered together in a well-supported terminal clade (ML bootstrap = 100%) and were considered to represent a new species described here as *Phytophthora mediterranea* sp. nov. (Figure 2).

Phylogenetically, the new *Phytophthora* species is closely related to *P. cinnamomi*, from which it can be distinguished on the basis of 15, 23 and 49 bp in ITS, Btub and *cox*1 loci, respectively. The other evolutionarily closest species, *P. parvispora* differs by a total of 105 nucleotides in the investigated DNA regions.

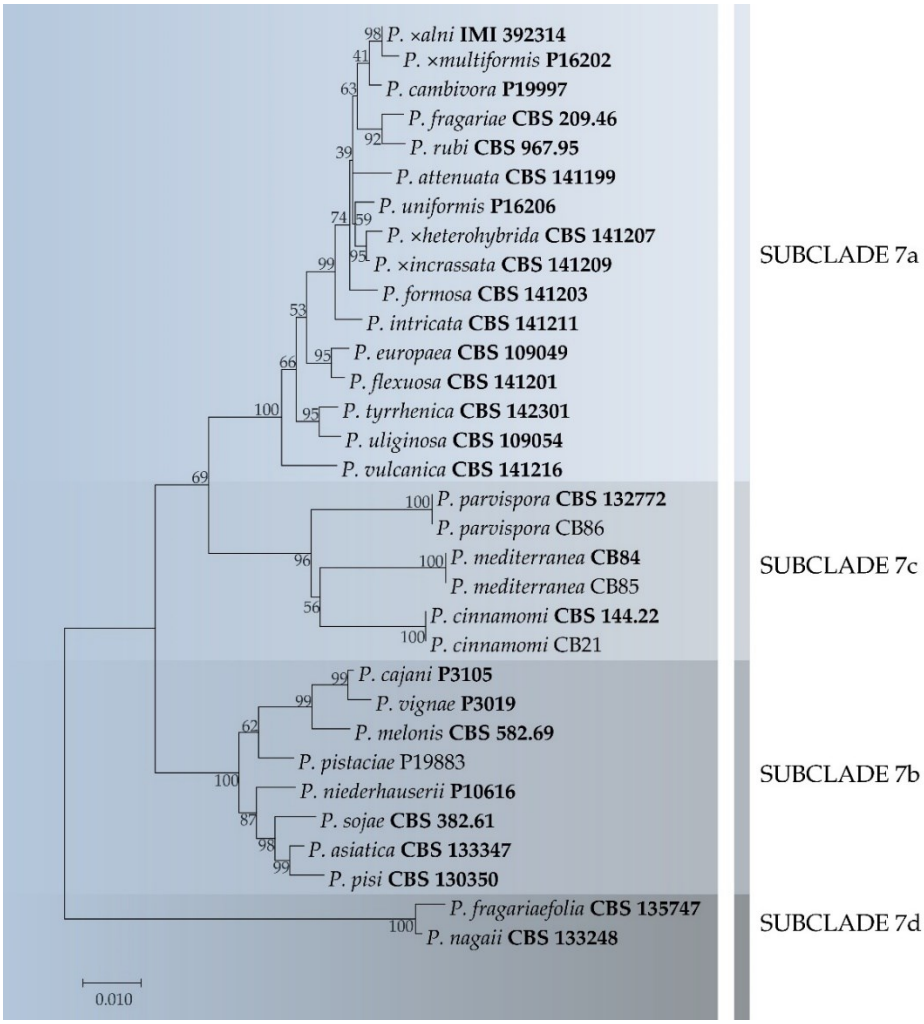

Figure 2. Maximum likelihood tree obtained from combined ITS, Btub and *cox1* sequences of *Phytophthora* species belonging to clade 7. Data are based on the general time reversible model. A discrete gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap support values in percentage (1000 replicates) are given at the nodes. Ex-type cultures are reported in bold.

3.4. Taxonomy

Phytophthora mediterranea Bregant, Mulas and Linaldeddu sp. nov. (Figure 3).

MycoBank: MB839612.

Etymology: the epithet refers to the Mediterranean region, where the species was originally discovered.

Holotype: CBS H-24768.

Host/distribution: potted *Myrtus communis* plants with root rot symptoms in Italy.

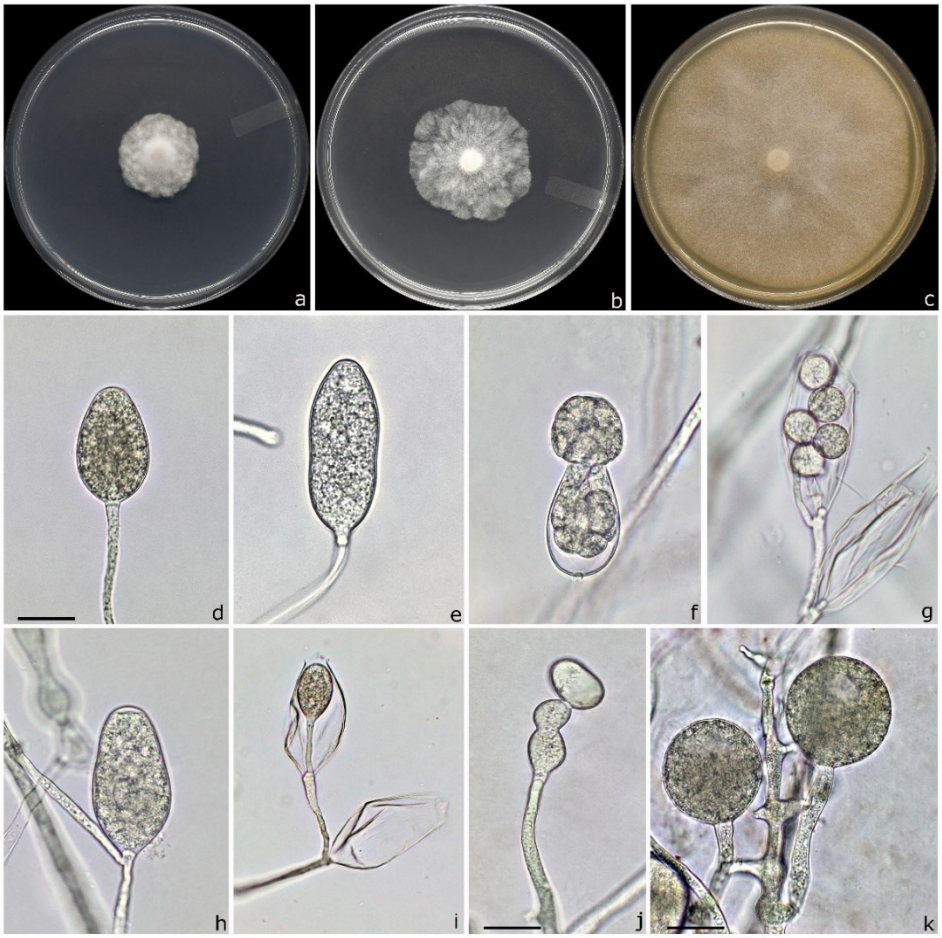

Figure 3. Colony morphology of *Phytophthora mediterranea* (CB84) after 7 days growth at 25 °C on potato dextrose agar (PDA) (a), malt extract agar (MEA) (b) and carrot agar (CA) (c). Sporangia observed in pond water: non-papillate, persistent and ovoid (d), elongated (e), releasing zoospores (f,g). External (h) and internal proliferations (i), hyphal swellings (j) and terminal chlamydospores (k). Scale bars = 20 μm.

Description: sporangia were abundantly produced in unsterile pond water after 36-48 h of incubation at 20 °C. They were non-papillate, rarely semi-papillate, persistent, from ovoid to ellipsoid and less frequently elongated or distorted (Figure 3d,e,h). Typically, sporangia were borne terminally on unbranched sporangiophores and only occasionally on compound sporangiophores. Sporangial proliferation was usually external and very rarely internal (nested and extended) (Figure 3g–i).

Sporangia size ranged from 27.2 to 64.9 μm in length (av. 42.9 ± 9.3 μm) and from 20.1 to 46.8 μm in breadth (av. 29.8 ± 5.3 μm) with a length/breadth ratio of 1.4 ± 0.1 (n = 50). Zoospores were abundantly produced in liquid cultures after 36-48 h (Figure 3f,g). Globose to sub-globose, irregular and catenulate hyphal swellings were abundantly produced on CA (Figure 3j). Spherical chlamydospores were mostly terminal and only occasionally lateral and intercalary and were abundantly produced on CA and water (Figure 3k). Chlamydospores ranged from 14.9 to 37.5 μm in diameter (av. 24.5 ± 5.0 μm). No gametangia were observed on pure cultures on CA suggesting a heterothallic behaviour.

Cultural characteristics: colonies were stellate on PDA and radiate on MEA whereas on CA showed a regular margin with a cottony and aerial mycelium

without a distinct pattern. On PDA and MEA colony growth was slow, whereas on CA colony reached 80 mm diameter in 7 d at 25 °C.

Cardinal temperatures: *minimum* <10 °C, *maximum* >37 °C and *optimum* 32 °C. Both isolates failed to grow at 40 °C and mycelium did not resume growth when plates were moved to 20 °C.

Material examined: ITALY: Oristano, isolated from roots of a potted plant of *Myrtus communis*, 18 April 2019, isolated by Antonio Mulas, HOLOTYPE CBS H-24768, a dried culture on CA, culture ex-holotype CB84 = CBS 147720. ITALY: Oristano, isolated from rhizosphere and fine roots of a potted plant of *Myrtus communis*, 12 November 2020, isolated by Carlo Bregant (culture CB85).

Notes: *Phytophthora mediterranea* differs from the closely related species *P. cinnamomi* and *P. parvispora* through a combination of unique morphological characters and molecular data such as sporangia shape and sizes, growth pattern on different culture media, a higher *maximum* and *optimum* temperature value for growth (Figure 4) as well as a total of 87 (*P. cinnamomi*) and 105 (*P. parvispora*) fixed differences in the ITS, Btub and *cox*1 sequences.

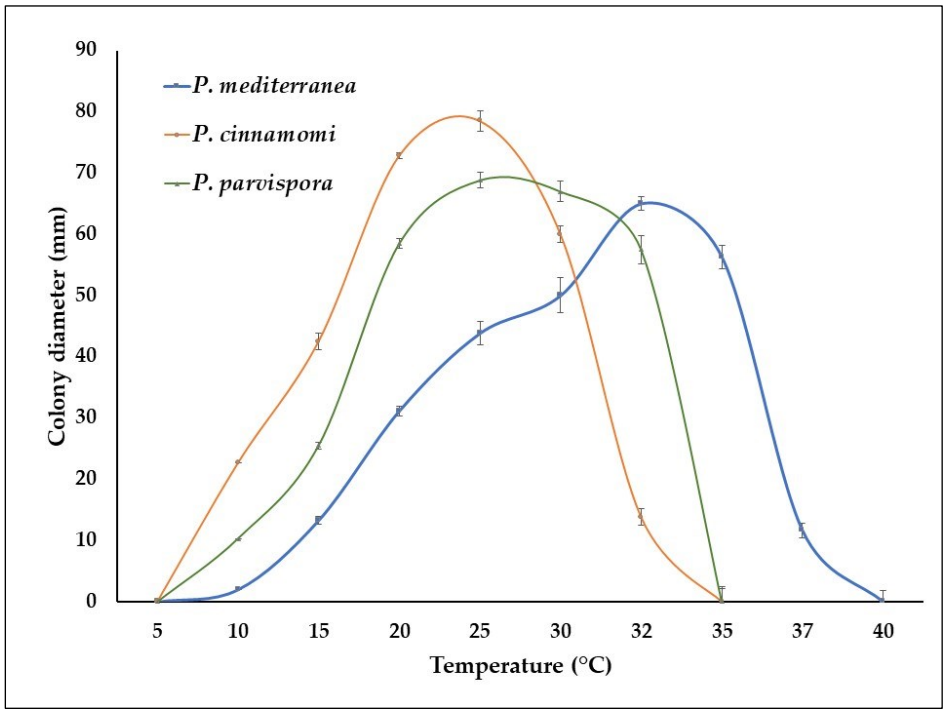

Figure 4. Mean colony diameter (± standard deviation) of *P. cinnamomi* (CB21), *P. mediterranea* (CB84) and *P. parvispora* (CB86) after 96 h on CA in the dark at different temperatures.

3.5. Pathogenicity Test

In artificial inoculation trials, *P. mediterranea* was shown to be pathogenic on myrtle and lentisk seedlings. After 20 days from inoculation, all plants inoculated with *P. mediterranea* displayed necrotic inner bark lesions that spread up and down from the inoculation site (Figure 5). On myrtle, the necrotic lesions caused by the isolate of *P. mediterranea* were significantly larger than those caused by *P. cinnamomi*; in contrast on lentisk, *P. cinnamomi* showed itself to be more aggressive than *P. mediterranea* (Table 4).

Control plants inoculated with sterile CA plugs remained symptomless, only a light brown discoloration limited to the inoculation point was observed on lentisk seedlings (Figure 5). Both *Phytophthora* species were successfully re-

isolated from all inoculated plants, fulfilling Koch's postulates. No *Phytophthora* isolates were re-isolated from control plants.

Table 4. Mean lesion length (cm) (± standard deviation) caused by *Phytophthora mediterranea* (CB84) and *P. cinnamomi* (CB21) on myrtle (M) and lentisk (L) seedlings and percentage of positive re-isolations.

Species	Myrtle *	Lentisk *	Re-Isolation Frequency (%)
Phytophthora mediterranea	1.01 ± 0.4a	1.9 ± 0.6b	100 (M) and 100 (L)
Phytophthora cinnamomi	0.68 ± 0.3b	2.3 ± 0.8a	100 (M) and 100 (L)
Control	-	0.5 ± 0.2c	-

* Values in column with the same letter do not differ significantly at $p = 0.05$, according to LSD multiple range test.

Figure 5. Symptoms observed on myrtle and lentisk seedlings 20 days after inoculation with *Phytophthora mediterranea* (a,d) and *P. cinnamomi* (b,e). Control seedlings (c,f).

4. Discussion

The results obtained allowed us to expand knowledge on the diversity of *Phytophthora* species in Italian forest nurseries. The investigated nurseries are located in sites with very different climatic conditions, ranging from the Mediterranean (N2) to alpine ones (N3), including an intermediate climate

typical of temperate hilly and low mountain areas (N1 and N4). These differences were also reflected in the plant species cultivated, which were typical of the different geographic regions.

Over the past two decades, diseases caused by *Phytophthora* species have been reported in a wide range of economically important ornamental plants worldwide and are considered yield-limiting factors in nursery production [**13**,**29**].

Our findings demonstrated that all the investigated nurseries were severally impacted by *Phytophthora* diseases. The N1 in particular was the most affected by the problem, and basically all cultivated plant species typical of the Mediterranean region were affected by *Phytophthora* infections.

In total, 15 *Phytophthora* species were isolated in pure culture from 14 hosts and identified on the basis of morphological features and DNA sequence analysis. Nine *Phytophthora* species were isolated in N1, five in N2, two in N4 and one in N3. The species obtained are representative of seven phylogenetic clades and included 14 previously known species: *P. acerina*, *P. bilorbang*, *P. cactorum*, *P. cinnamomi*, *P. citrophthora*, *P. megasperma*, *P. nicotianae*, *P. palmivora*, *P. parvispora*, *P. pistaciae*, *P. plurivora, P. pseudocryptogea, P. pseudosyringae* and *P. psychrophila*. In addition, two isolates could not be assigned to any known species and are therefore described here as *P. mediterranea* sp. nov.

Phytophthora mediterranea was shown to be associated with collar and root rot symptoms of myrtle seedlings. Phylogenetically, *P. mediterranea* is closely related to *P. cinnamomi* but the two species differ in 87 nucleotides in the three DNA regions studied. Morphologically *P. mediterranea* can be easily distinguished from *P. cinnamomi* on the basis of its smaller sporangia, colony growth pattern and higher *optimum* and *maximum* temperature values. Data from the pathogenicity tests showed that *P. mediterranea* has the potential to threaten the native Mediterranean maquis vegetation. A study is currently in progress to evaluate the susceptibility of the main Mediterranean maquis species to this new pathogen (Linaldeddu, unpublished data). The high *optimum* temperature value for growth of 32 °C and the high production of long-term survival propagules (chlamydospores) suggest that *P. mediterranea* is well adapted to survive in Mediterranean environments.

Phytophthora mediterranea belongs to clade 7c together with *P. cinnamomi* and *P. parvispora,* which were also obtained in this study. *Phytophthora cinnamomi* was the dominant species as it has been detected on five different hosts confirming its well-known polyphagous nature [**11**,**58**], and in three of the four monitored nurseries. The three nurseries are characterized by different environmental conditions ranging from the Mediterranean climate of site 2 located in Sardinia to the cold habitat of site 3 in the pre-Alps. This aspect emphasizes the potential of *P. cinnamomi* to survive in very different environments including low temperature habitats, as confirmed by a recent study in which this pathogen was detected in an outdoor blueberry stand in Germany [**59**]. Cold regions such as alpine and sub-alpine regions were long considered *P. cinnamomi* free, due to the inactivity of this species in soil at temperatures below 10 °C [**60**]. Analysis on current distribution data, global change and a forecast model, have allowed a global map to be created, identifying the cold areas of the planet as those at greatest risk of introduction and spread of *P. cinnamomi* in the future [**61**]. The discovery of *P. cinnamomi* in mountain nurseries in Italy confirms this trend and poses the risk of diffusion of this pathogen on new susceptible hosts among the alpine species.

Phytophthora nicotianae was the second dominant species. It has been isolated from four host species confirming the results of previously studies on

the role of the nursery trade as one of the main ways of spreading for this pathogen [**48**,**62**]. The other twelve species have been isolated from a single plant host and only one nursery. Differences in species diversity among nurseries are difficult to interpret. It is plausible that different agronomical practices including sanitation, water management, origin and treatment of seeds as well as host plant diversity could have a role in the differences observed in terms of *Phytophthora* assemblages. Some species such as *P. parvispora* and *P. pistaciae* have confirmed a specificity towards some plant hosts already reported in other studies [**49**,**50**]. *Phytophthora parvispora*, long considered a variety of *P. cinnamomi*, is closely linked to *Arbutus unedo* plants in both nursery and natural areas of the Mediterranean Basin [**49**]. It has also recently been reported in North American nurseries associated with seedlings destined for reforestation programmes and in pomegranate orchards in Turkey [**63**,**64**]. *Phytophthora pistaciae* is a species characterized by a very limited geographic distribution and host range; it was originally described in Iran associated with *Pistacia vera* gummosis [**65**] and more recently in Italy on potted *Pistacia lentiscus* seedlings [**50**].

The other species, except *P. acerina*, have already been reported in different nurseries in Europe, Australia and North America on several plant hosts [**34**,**39**,**48**,**63**]. Nonetheless, seven new *Phytophthora*–host associations were detected in this study: *P. psychrophila*/*Ilex aquifolium*, *P. pseudosyringae*/*I. aquifolium*, *P. pseudocryptogea*/*Laurus nobilis*, *P. megasperma*/*L. nobilis*, *P. citrophthora*/*L. nobilis*, *P. bilorbang*/*Phyllirea latifolia* and *P. palmivora*/*P. latifolia.*

Phytophthora acerina was originally described in Northern Italy from declining maple trees [**66**] and more recently on declining olives with sudden death symptoms [**67**]. Its reports have increased in the last years in both natural and agricultural environments in Italy and its role in the onset of sudden death and dieback of kiwi, walnut and pomegranate orchards as well as oaks, chestnut and ash forests has been ascertained (Linaldeddu, unpublished data). In a nursery, *P. acerina* appears to be a very rare species, this is the first report on nursery plants in Europe, but it was recently reported in nursery plants in California [**68**]. The discovery of *P. acerina* and *P. plurivora* on seedlings potentially destined to reforestation programmes highlights the risk of further spread of these species, directly associated with the decline of grey alder on the Alps [**51**].

5. Conclusions

The findings obtained in this study highlight the occurrence of multiple *Phytophthora* species in Italian forest nurseries. Many of the species isolated are common in Italian nurseries, while others are rare or have never been reported. *Phytophthora pistaciae* is an example of a rare and possibly exotic species with a high risk of spread to the Mediterranean maquis. In addition, a new species closely related to *P. cinnamomi* and *P. parvispora* was isolated from potted myrtle plants. The myrtle is a typical shrub of the Mediterranean Basin, and Sardinia is recognized as one of its main local centres of diversity [**69**]. Its fruit and leaves exhibit antioxidant, antibacterial and antifungal properties, as well as being used for their content of essential oils and most commonly as an ingredient in a locally made liquor [**70**]. Nursery plants are often used for the creation of new orchards [**71**]. The use of infected plants could represent an important pathway for *P. mediterranea.*

Finally, the discovery of *P. cinnamomi* in alpine nurseries confirms the progressive expansion of this species towards the coldest areas of the globe, probably driven by climate change. A survey is currently in progress to map the

distribution of *Phytophthora* species in alpine nurseries in Italy, with the purpose of evaluating the risk of diffusion of these pathogens through restoration and reforestation programmes.

Author Contributions: Conceptualization, B.T.L.; field survey, sample collection and assay, B.T.L., C.B., A.A.M., G.R. and G.P.; data analysis, B.T.L., C.B., G.R., A.D. and L.M.; funding acquisition B.T.L. and L.M; draft writing B.T.L. C.B and G.R.; review and manuscript editing, B.T.L., G.P., L.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by grant number DOR2103497/21 "Impatto delle specie invasive di *Phytophthora* sulla biodiversità delle formazioni forestali in Italia" and by the "Fondo di Ateneo per la ricerca 2020". Internal funding was provided by the University of Sassari.

Data Availability Statement: Data sharing not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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General discussion

Field surveys conducted in different Italian, Portuguese and Slovenian forests over a three-year period showed a complex of 26 pathogenic *Phytophthora* species associated with leaf and shoot blights, bleeding cankers and root rot symptoms on 41 plant species typical of mountain vegetation. The complex of symptoms observed were compatible with both air and soil-borne *Phytophthora* infections. The extensive field surveys conducted in this study highlight that severe disease outbreaks and mortality events are currently affecting shrubs and trees especially along riparian habitats from the plain up to above the tree line (>2000 m. a.s.l.).

Among the main plant species threatened by *Phytophthora* attacks, many belong to the *Betulaceae*, *Cupressaceae*, *Ericaceae*, *Fagaceaee*, *Pinaceae*, *Rosaceae*, *Salicaceae*, and *Sapindaceae* families. In particular, the extensive mortality observed on the three main alder species widespread in Italy is particularly alarming.

Overall, a surprisingly high diversity of *Phytophthora* in mountain ecosystems emerges from this study. This includes both cosmopolitan and polyphagous pathogens and rare species characterized that attack a limited number of plant hosts in a few geographic areas. Results show that many *Phytophthora* species chiefly belonging to clades 1 and 3 are characterized by an aerial lifestyle because they are able to produce caducous sporangia. In particular, *P. pseudosyringae* was the species more conspicuously isolated from a huge number of hosts in all monitored sites. This pathogen represents the key species involved in aerial infections on high-altitude small shrubs and heaths such as dwarf pine, alpine junipers, rhododendrons, small willow formations and blueberry. These shrubs are characterized by a preferential creeping behaviour and very limited heights above the ground. This aspect can help infections by *Phytophthora* sporangia and zoospores spreading from the soil. *Phytophthora pseudosyringae* have previously been reported in different continents in cold environments (Jung *et al*., 2003; Hwang *et al*., 2007; Varela *et al*., 2007; Fajardo *et al*., 2017; Redondo *et al*., 2018; Bregant *et al*., 2020). Many important aspects regarding the infectivity, epidemiology and survival of *P. pseudosyringae*, in subalpine areas remain to be understood. Currently, further studies are ongoing to clarify the lifecycle of this pathogen in relation to the snow cover and soil temperature during winter. The layer between soil and snowpack, occupied by litter, could represent an important substrate during winter. Many of the species isolated above the tree line, are able to produce chlamydospores and catenulate hyphal swellings. The production of chlamydospores has also been ascertained for the first time for *P. pseudosyringae.*

In addition to high mountain vegetation, the extensive studies conducted along riparian formations and streams showed that *P. plurivora* is the main pathogen involved in the decline of riparian vegetation. In addition to *P. plurivora,* other two closely-related species of the ex *P. citricola* complex

emerged in riparian systems of Italy and Portugal, *P. acerina* and *P. multivora*. The distribution of *P. acerina* appeared limited to Italy in both natural ecosystems and crops (Ginetti *et al*., 2014; Bregant *et al.*, 2020; Linaldeddu *et al*., 2023). The increasingly frequent reports on the spread of this species in Northern Italy and its absence in the rest of Europe suggest that further studies are needed to understand the origin of this pathogen, probably originating from Northern Italy. On the other hand, until now there have been few reports on *P. multivora* in Italy and Europe (Mrazkova *et al*., 2013; Tsykun *et al*., 2022). This pathogen represents one of the most invasive and destructive in Australia, causing dieback and death of hundreds plant species (Scott *et al*., 2009; Burgess *et al*., 2017a; Migliorini *et al*., 2019). The discovery of *P. multivora* in riparian habitats in Portugal is of particular concern.

Finally, the study conducted in four Italian forest nurseries ranging from the Mediterranean to the Alps highlight an alarming scenario linked to the occurrence of several invasive pathogens. Of the 15 species obtained from nursery plants, *P. cinnamomi* was the dominant species, as it has been detected on five different hosts, confirming its well-known polyphagous nature. The occurrence of this species was critical in a mountain nursery of the Italian Pre-Alps, affecting beech and fir seedlings. This aspect emphasizes the potential of *P. cinnamomi* to survive in very different environments including low temperature habitats, due to its ability to produce chlamydospores (Mc Dougall *et al*., 2003; Khaliq *et al*., 2020; Gyeltshen *et al*., 2021; Serrano *et al*., 2022). The expansion of this pathogen towards colder areas could be favoured by climate change (Burgess *et al*., 2017b; Khaliq *et al*., 2020, 2021). A new species closely to *P. cinnamomi,* was also discovered from nursery plants and described as *P. mediterranea*. The very high *optimum* temperature value for growth (32 °C) and the abundant production of long-term survival propagules (chlamydospores) makes *P. mediterranea* a serious risk for Mediterranean vegetation.

In addition to *P. mediterranea*, the research conducted allowed another two new species to be discovered and described: *P. heteromorpha* and *P. pseudogregata* sp. nov.

Phytophthora pseudogregata have been isolated from aerial infections and rhizosphere of subalpine shrubs such as common juniper, green alder and rhododendron, and show a moderate pathogenicity on alpine juniper. Whereas *P. heteromorpha* have been obtained from rhizosphere and water of grey alder riparian formations. *Phytophthora heteromorpha* and *P. pseudogregata* show a wide thermal range for growth, suggesting a high plasticity in different environmental conditions.

Both species belong to the phylogenetic clade 6, one rich in species and well studied. It contains several species described recently, such as *P. amnicola*, *P. borealis*, *P. chlamydospora, P. bilorbang*, *P. crassamura, P. fluvialis*, *P. gibbosa, P. gregata, P. heteromorpha, P. lacustris, P. litoralis, P. mississippiae, P. moyootj, P. ornamentata, P. pinifolia, P. pseudogregata, P. riparia* and *P.*

thermophila (Abad *et al*., 2023). The majority of species in this subclade, including *P. heteromorpha* and *P. pseudogregata*, have been described in forest ecosystems.

Overall, the results obtained have contributed to expanding scientific knowledge about the diversity of *Phytophthora* in mountain forests. Italy is a long peninsula characterized by extremely varied climatic and vegetation conditions. The mountainous hinterland and surrounding areas are strongly related to the Mediterranean Sea on every side. The stretched disposition along different climatic zones and the very diversified morphology make Italy a huge biodiversity hotspot and the first country in Europe in terms of number of living species (Zurlini *et al*., 1999; Myers *et al*., 2000; Coll *et al*., 2010). To date, *Phytophthora* research in Italy has been conducted chiefly in temperate and Mediterranean areas; most of the studies are concentrated in central and southern Italy, where several *Phytophthora* species have been reported, in particular on the two major islands: Sardinia (31 species) and Sicily (22 species). This is mainly due to the greater scientific interest linked to the occurrence of mortality phenomena in these areas starting from the last 15-20 years. In Northern Italy, before this study little was known about the diffusion of *Phytophthora* species in forest ecosystems, with only a few reports relegated to chestnut and oak trees (Jung *et al*., 1999; Vettraino *et al*., 2001, 2005).

The results obtained have contributed to expanding knowledge about the number of *Phytophthora* species occurring in Northern Italy. In particular, 24 species of *Phytophthora* are now reported in the Veneto Region (Fig. 4).

Overall, 44 species of *Phytophthora* are currently reported in natural ecosystems of Italy. Among them, twelve species (*P. bilorbang*, *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citricola*, *P. lacustris*, *P. megasperma*, *P. multivora*, *P. plurivora*, *P. pseudocryptogea*, *P. quercina* and *P. syringae*) are present throughout the peninsula.

The structures of *Phytophthora* communities varied in accordance with the ecology and climatic *optimum* of the various species. Considering the northern part of the country, nine *Phytophthora* species were found to be exclusive to this area, a sign that the low temperatures on the Alps allow the survival of a large community of species suited to colder climates.

Concluding remarks

Phytophthora diseases on Mediterranean species have been getting the most attention in the last decades; in this study an alarming scenario of emerging and new *Phytophthora* diseases emerges from mountain vegetation, with many species such as *Alnus incana*, *Alnus viridis*, *Betula pubescens*, *Rhododendron ferrugineum*, *Salix caprea* and *Vaccinium myrtillus* threatened by invasive *Phytophthora* species. In particular, the wide distribution of *P. plurivora* was confirmed on riparian

vegetation whereas *P. pseudosyringae* was the species more diffuse at the highest altitude on sub-alpine vegetation. The current trend of discovering an increasing number of pathogenic species in forest ecosystems emphasizes how much more we need to learn about *Phytophthora* biodiversity and their impact on natural ecosystems. In general, this study contributes to expanding knowledge on the ecology of *Phytophthora* species in both natural areas and nurseries with 87 new host-pathogens associations and 10 new *Phytophthora* reports for Italy, 11 for Portugal and 6 for Slovenia.

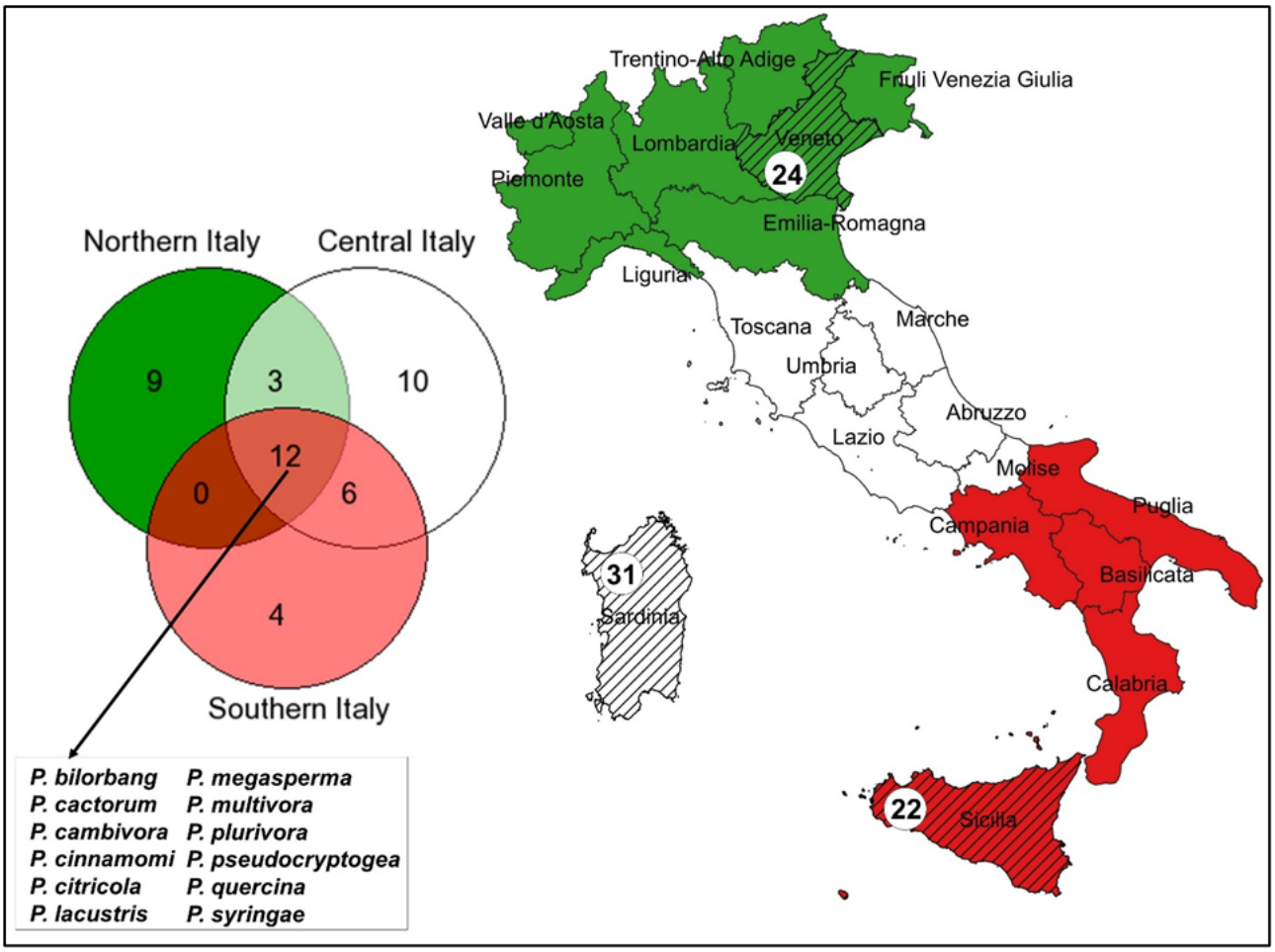

Figure 4. Distribution map and Venn diagram of *Phytophthora* spp. in Italian forest ecosystems. Different colours represent the three Italian zones: red = Southern Italy; white = Central Italy; green = Northern Italy. The regions within each zone with the highest *Phytophthora* diversity are indicated by stripes.

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Acknowledgement

I would like to acknowledge all members of the forest pathology team at the University of Padova, and in particular for Prof. Lucio Montecchio and my friends and colleagues Giovanni Rossetto and Nicolò Sasso, for their collaboration, precious suggestions and help during these three years.

I am sincerely grateful also to Prof. Artur Alves, Dr. Eduardo Batista, Dr. Nikica Ogris and Dr. Barbara Piškur for their support and our productive scientific collaboration during my periods abroad. Finally, thanks to my family, to encourage and believe me every day.

List of publications linked to the thesis

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