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Diversity and Distribution of *Phytophthora* Species in Protected Natural Areas in Sicily

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Abstract: The aim of this study was to investigate the occurrence, diversity, and distribution of *Phytophthora* species in Protected Natural Areas (PNAs), including forest stands, rivers, and riparian ecosystems, in Sicily (Italy), and assessing correlations with natural vegetation and host plants. Fifteen forest stands and 14 rivers in 10 Sicilian PNAs were studied. *Phytophthora* isolations from soil and stream water were performed using leaf baitings. Isolates were identified using both morphological characters and sequence analysis of the internal transcribed spacer (ITS) region. A rich community of 20 *Phytophthora* species from eight phylogenetic clades, including three new *Phytophthora* taxa, was recovered (17 species in rhizosphere soil from forest stands and 12 species in rivers). New knowledge about the distribution, host associations, and ecology of several *Phytophthora* species was provided.

Keywords: soilborne pathogens; invasive species; natural ecosystems; streams; vegetation type; baiting; ITS region

1. Introduction

Due to its location in the central Mediterranean Sea and its vast area of 25.708 km^2, Sicily is one of the most important biodiversity areas in Europe and in the Mediterranean basin [1,2] harboring more than 3000 plant species [3], 321 of which are endemic to Sicily [4]. Sicily's outstanding floristic and ecological diversity was acknowledged by the establishment of numerous Protected Natural Areas (PNAs; Italian National Law 394/91), including three Regional Parks, 72 Regional Natural Reserves and 223 Sites of Community Importance (Habitats Directive 92/43/EEC).

During a recent monitoring of the health conditions of oak and beech trees in forests of the Etna, Madonie, and Nebrodi Regional Parks in Sicily (southern Italy), severe symptoms of crown decline were observed, indicating fine root losses caused by soilborne pathogens from the genus

Phytophthora [5]. With more than 150 described species grouped in twelve multigenic phylogenetic Clades [6], this oomycete genus comprises some of the most aggressive plant pathogens of forests and other natural ecosystems [7–16]. Several studies highlighted the diversity of *Phytophthora* species in native vegetation and their potential impact on natural ecosystems [17–26]. The presence of exotic, potentially invasive *Phytophthora* species often represents a threat for the survival of native plant species and may alter the stability of the entire ecosystem. In Sardinia, a survey in the National Park of the La Maddalena archipelago demonstrated the involvement of exotic *Phytophthora* species in the widespread mortality of *Quercus ilex* trees and Mediterranean maquis vegetation [25,27,28]. Outplanting of infected nursery stock is considered a primary pathway for the introduction of non-native *Phytophthora* species into forest ecosystems [10,29–36]. In recent years, great attention has been paid to surface water as a source of *Phytophthora* inoculum in natural ecosystems. Surveys of rivers, streams, and riparian ecosystems in several continents have revealed a huge diversity of *Phytophthora* species, including primarily aquatic species which are considered as opportunistic pathogens, but also soilborne and airborne primary pathogens [8,20,21,37–41]. However, all *Phytophthora* species have the potential to be disturbance factors in natural ecosystems, in particular, those of exotic origin, provided that the environmental conditions are conducive to disease development [9,10,32]. The number of species known in the genus *Phytophthora* has increased dramatically during the past decade, mainly due to extensive surveys in previously unexplored ecosystems such as natural forests, riparian ecosystems, streams, and irrigation systems [6,20,21,25,42,43]. The ecological role of most of these new species and their distribution in natural ecosystems are still largely unknown, although the knowledge of the *Phytophthora* community and its potential impact on native vegetation is a prerequisite for proper management of PNAs. Despite the large number of *Phytophthora* species reported from nurseries and agricultural crops in Sicily, their occurrence and ecology in natural environments have received little attention. The aims of this study were to examine (i) the diversity and distribution of *Phytophthora* species in forest stands and river systems of Sicilian PNAs and (ii) their association with natural vegetation and potential host plants.

2. Materials and Methods

2.1. Sampling and Phytophthora Isolation

Ten PNAs in northern and eastern Sicily, including the three Regional Parks (RP), five Regional Nature Reserves (RNR), and two Sites of Community Importance (SCI), characterized by different ecological conditions were included in this study (Table S1, Figure S1). Twenty sites in 15 characteristic Sicilian forest stands (FS) in seven PNAs and 14 rivers running through nine PNAs were included in the survey of distribution and diversity of *Phytophthora* species (Table 1 and 2, Figures 1 and 2). Sampling activities were carried out during the spring of 2013 and 2015.

In total, 83 rhizosphere soil samples from mature specimens of 17 tree species were collected in the 15 forest stands (Table 1). Soil sampling and isolation methodologies were performed according to Jung [9]. Subsamples of ca. 200 mL soil were used for baiting tests at 18–20 °C in a walk-in growth chamber with 12 h natural daylight. Young leaves of native species (mainly *Ceratonia siliqua* and *Quercus* spp.) were used as baits floated over flooded soil. Necrotic segments (2 × 2 mm) from Infected leaves were plated onto selective PARPNH-agar [11]. Petri dishes were incubated at 20 °C in the dark. Outgrowing *Phytophthora* hyphae were transferred onto V8-juice agar (V8A) under the stereomicroscope. *Phytophthora* isolations from rivers were performed using an in situ baiting technique [21]. At each site, 10 non-wounded young leaves of *C. siliqua*, *Quercus* spp. and *Citrus* spp. were placed in a mesh-bag styrofoam raft (25 × 30 cm) [21] rigged to float on the water surface. In total 35 rafts were placed in 14 rivers (Table 2, Figure 2) and collected after 3–5 days. Isolations from necrotic leaf lesions were carried out as described before. All obtained isolates were maintained on V8A and stored at 6 °C in the dark.

Table 1. Vegetation, geological substrate, municipality, geographic coordinates, and altitude of the 15 forest stands sampled in seven Protected Natural Areas in Sicily, tree species sampled and *Phytophthora* taxa isolated.

Forest Stand (FS) No.	Protected Natural Area [a]	Vegetation (Natura 2000 Code, Forest Stand Type, Phytocoenosis) [b,c,d]	Geological Substrate	Municipality	Sampling Site No.	Geographic Coordinates (DATUM WGS84)	Altitude (m a.s.l.)	Sampled Tree Species (No. of *Phytophthora*-Positive Soil Samples/Sampled Trees)	*Phytophthora* spp. (No. of Positive Soil Samples) [f]
FS-1	Etna RP	Natura 2000 CODE: 9340. Forest stand type: Meso-Mediterranean evergreen oak forest. Phytocoenosis: *Teucrio siculi-Quercetum ilicis* subass. *Teucrietosum siculi*.	Volcanic (Alkali Basalt-Na)	Zafferana Etnea (CT)	I	37°41′44.53″ N–15°05′00.04″ E	1030	*Quercus ilex* L. (3/5)	MUL (1); QUE (2)
					II	37°41′05.94″ N–15°05′13.04″ E	890	*Quercus pubescens* Willd. s. l. (4/5)	PSY (4) [g]
					V	37°41′53.92″ N–15°06′01.05″ E	660	*Q. pubescens* s. l. (4/5)	CAM (1); QUE (3)
FS-2	Etna RP	Natura 2000 CODE: 91M0. Forest stand type: Supra-Mediterranean turkey oak forest. Phytocoenosis:*Vicio cassubicae-Quercetum cerridis*.	Volcanic (Alkali Basalt-Na)	Sant'Alfio (CT)	III	37°46′26.02″ N–15°05′37.23″ E	1345	*Q. pubescens* s. l. (3/6)	PSY (3) [h]
FS-3	Etna RP	EUNIS CODE: G1.916 [e]. Forest stand type: Supra-Mediterranean birch forest. Phytocoenosis: Aggregation with *Betula aetnensis*.	Volcanic (Alkali Basalt-Na)	Sant'Alfio (CT)	IV	37°46′14.90″ N–15°03′34.56″ E	1667	*Betula aetnensis* Raf. (0/1)	-
FS-4	Etna RP	Natura 2000 CODE: 9220. Forest stand type: Supra-Mediterranean beech forest. Phytocoenosis: *Epipactido meridionalis-Fagetum sylvaticae*.	Volcanic (Alkali Basalt-Na)	Castiglione di Sicilia (CT)	X	37°48′50.94″ N–15°01′24.42″ E	1874	*Fagus sylvatica* L. (1/1)	VUL (1)
FS-5	Nebrodi RP	Natura 2000 CODE: 9210. Forest stand type: Supra-Mediterranean beech forest. Phytocoenosis: *Anemono apenninae-Fagetum sylvaticae*.	Sedimentary–M. Soro Flysh (Marly claystones and limestones, grading upward to quarzarenites)	Militello Rosmarino (ME)	VI	37°56′22.20″ N–14°40′15.49″ E	1450	*F. sylvatica* (5/7)	CAM (4); MEG (1) [i,j]
								Q. pubescens s. l. (1/1)	CAM (1)
				Cersarò (ME)	IX	37°55′40.90″ N–14°41′35.48″ E	1783	*F. sylvatica* (1/3)	CAM (1)
FS-6	Nebrodi RP	Natura 2000 CODE: 9340. Forest stand type: Meso-Mediterranean evergreen oak forest. Phytocoenosis: *Teucrio siculi-Quercetum ilicis*.	Sedimentary–M. Soro Flysh (Marly claystones and limestones, grading upward to quarzarenites)	San Fratello (ME)	VII	37°57′16.38″ N–14°37′18.34″ E	1050	*Q. ilex* (3/5)	CAM (1); GON (2); PSY (1) [i]
FS-7	Nebrodi RP	Natura 2000 CODE: 91M0. Forest stand type: Meso-Mediterranean turkey oak forest. Phytocoenosis: *Arrhenathero nebrodensis-Quercetum cerridis*.	Sedimentary–M. Soro Flysh (Marly claystones and limestones, grading upward to quarzarenites)	Randazzo (CT)	VIII	37°56′40.81″ N–14°54′17.89″ E	1420	*F. sylvatica* (1/1)	CAM (1) [i]
								Quercus cerris L. (1/1)	CAM (1) [i]

Table 1. *Cont.*

Forest Stand (FS) No.	Protected Natural Area [a]	Vegetation (Natura 2000 Code, Forest Stand Type, Phytocoenosis) [b,c,d]	Geological Substrate	Municipality	Sampling Site No.	Geographic Coordinates (DATUM WGS84)	Altitude (m a.s.l.)	Sampled Tree Species (No. of *Phytophthora*-Positive Soil Samples/Sampled Trees)	*Phytophthora* spp. (No. of Positive Soil Samples) [f]
FS-8	Nebrodi RP	Natura 2000 CODE: 9330. Forest stand type: Meso-Mediterranean cork oak forest. Phytocoenosis: *Genisto aristatae-Quercetum suberis*.	Sedimentary–Numidian Flysch (quarzarenites and clays)	Geraci Siculo (PA)	XVII	37°53′22.33″ N–14° 8′10.77″ E	710	*Quercus suber* L. (2/2)	GON (2); MEG (1)
FS-9	Madonie RP	Natura 2000 CODE: 9380. Forest stand type: Supra-Mediterranean holly forest. Phytocoenosis: *Ilici aquifoliae-Quercetum austrotyrrhenicae*.	Sedimentary–Numidian Flysh (quarzarenites and clays)	Petralia Sottana (PA)	XVIII	37°53′46.39″ N–14°3′55.22″ E	1390	*Ilex aquifolium* L. (1/1)	CAM (1)
FS-10	Madonie RP	Natura 2000 CODE: 91AA. Forest stand type: Meso-Mediterranean *Quercus pubescens* forest. Phytocoenosis: *Qurcetum leptobalani*.	Sedimentary–Numidian Flysh (quarzarenites and claystones)	Castelbuono (PA)	XIX	37°53′51.02″ N–14°3′58.77″ E	1412	*Q. pubescens* s. l. (1/3)	CAM (1)
FS-11	Madonie RP	Natura 2000 CODE: 9380. Forest stand type: Meso Mediterranean evergreen oak and holly forest. Phytocoenosis: *Geranio versicoloris-Quercetum ilicis*.	Sedimentary–Numidian Flysh (quarzarenites and claystones)	Castelbuono (PA)	XX	37°54′20.46″ N–14°4′29.39″ E	1110	*I. aquifolium* (0/3)	- [g]
								Q. ilex (2/4)	QUE (1); TYR (1); [g]
					XXI	37°54′50.19″ N–14°4′40.07″ E	850	*Castanea sativa* Mill. (1/2)	PLU (1)
FS-12	Pantalica RNR	Natura 2000 CODE: 92C0. Forest stand type: Thermo-Mediterranean riparian plane tree forest. Phytocoenosis: *Platano-Salicetum pedicellatae*.	Sedimentary (algal calcarenites and calcirudites)	Sortino (SR)	XI	37°07′48.0″ N–15°01′26.5″ E	236	*Populus nigra* L. (1/1)	PSC (1)
								Salix pedicellata Desf. (1/1)	PSC (1)
								Q. ilex + *Fraxinus oxycarpa* Bieb., mixed sample (1/1)	PSC (1); PLU (1); LAC (1)
								Platanus orientalis L. (1/1)	CAC (1); PLU (1)
								Ostrya carpinifolia Scop. (1/1)	LAC (1); PLU (1)
								P. orientalis + *Q. ilex*, mixed sample (1/1)	LAC (1); PLU (1)
					XII, XIV	37°08′19.3″ N–15°02′13.3″ E	221	*P. nigra* (1/1)	CAC (1); PLU (1) [k]
								Populus alba L. (1/1)	PSC (1); LAC (1); KEL (1)
								S. pedicellata (1/1)	PSC (1); LAC (1)
								Nerium oleander L. (1/1)	PLU (1)
								Celtis australis L. (1/1)	POL (1)
								Q. ilex (1/1)	PSC (1); PLU (1)
								P. orientalis (2/2)	CIP (1); LAC (2)
FS-13	Ciane RNR	Natura 2000 CODE: 92A0. Forest stand type: Thermo-Mediterranean riparian willow, poplar, and ash forest. Phytocoenosis: *Salicetum albo–pedicellatae*.	Alluvial sediments (loam and sandy limestone)	Siracusa (SR)	XIII	37°02′40.3″ N–15°14′40.7″ E	4	*F. oxycarpa* (4/4)	CRA (1); PSC (3); LAC (2); MEG (1); PLU (2);

Table 1. *Cont.*

Forest Stand (FS) No.	Protected Natural Area [a]	Vegetation (Natura 2000 Code, Forest Stand Type, Phytocoenosis) [b,c,d]	Geological Substrate	Municipality	Sampling Site No.	Geographic Coordinates (DATUM WGS84)	Altitude (m a.s.l.)	Sampled Tree Species (No. of *Phytophthora*-Positive Soil Samples/Sampled Trees)	*Phytophthora* spp. (No. of Positive Soil Samples) [f]
FS-14	Cavagrande RNR	Natura 2000 CODE: 92C0. Forest stand type: Thermo-Mediterranean riparian plane tree forest. Phytocoenosis: *Platano-Salicetum pedicellatae*.	Alluvial sediments (loam and sandy limestone)	Siracusa (SR)	XV	36°57′2.62″ N–15°11′8.15″ E	8	*Salix caprea* L. (2/2)	LAC (2); POL (1)
								P. orientalis (3/3)	CAC (1); PSC (3); MUL (2); PLU (1);
FS-15	Irminio SCI	Natura 2000 CODE: 92C0. Thermo-Mediterranean riparian plane tree forest. Phytocoenosis: *Platano-Salicetum pedicellatae*.	Sedimentary (calcarenites and marns)	Ragusa (RG)	XVI	37°00′1.9″ N–14°46′31.5″ E	430	*F. oxycarpa* (1/1)	PLU (1); PSC (1)
								Q. pubescens s. l. (2/2)	CIT (1); PLU (2)
								P. orientalis (1/1)	PLU (1)

[a] Etna RP = Etna Regional Park; Nebrodi-RP = Nebrodi Regional Park; Madonie-RP = Madonie Regional Park; Pantalica RNR = Pantalica, Valle dell'Anapo e Torrente Cavagrande Regional Natural Reserve (RNR); Ciane RNR = Fiume Ciane e Saline di Siracusa RNR; Cavagrande RNR = Cavagrande del Cassibile RNR; Irminio SCI = ITA080002—Alto corso del Fiume Irminio Site of Community Importance (SCI). [b] Vegetation features were in accordance with Natura 2000 sites data and respective management plans: ftp://ftp.minambiente.it/PNM/Natura2000/TrasmissioneCE_2016/schede_mappe/Sicilia/SIC_schede/. [c] Natura 2000 habitats: http://www.minambiente.it/sites/default/files/archivio/allegati/rete_natura_2000/int_manual_eu28.pdf. [d] *Teucrio siculi-Quercetum ilicis* subass. *Teucrietosum siculi*: Meso-Mediterranean acidophilous oak stand characterized by *Quercus ilex* L. mixed with calcifuge downy oaks (*Quercus dalechampii* and *Quercus congesta*). *Vicio cassubicae-Quercetum cerridis*: Supra-Mediterranean deciduous turkey oak stand characterized by *Quercus cerris* mixed with downy oaks (*Q. congesta* and *Q. delachampii*) *Fraxinus ornus* and *Acer obtusatum*. Aggregation with *Betula aetnensis*: Supra-Mediterranean pioneer vegetation dominated by the endemic *B. aetnensis* mixed with beech, turkey oak, and *Pinus nigra* subsp. *calabrica*. *Epipactido meridionalis-Fagetum sylvaticae*: Supra-Mediterranean beech forest dominated by *Fagus sylvatica*. *Anemono apenninae-Fagetum sylvaticae*: Acidophilous Supra-Mediterranean beech forest characterized by *F. sylvatica in* association in *Ilex aquifolium*. *Teucrio siculi-Quercetum ilicis*: Meso-Mediterranean acidophilous oak forest stand characterized by *Q. ilex* mixed with deciduous oaks (*Quercus virgiliana* and *Q. congesta*). *Arrhenathero nebrodensis-Quercetum cerridis*: Meso-Mediterranean acidophilous turkey oak forest stand typified by *Q. cerris*; at the higher altitude (ca. 1400 m) it is mixed with beech forest stands (*Anemono apenninae-Fagetum sylvaticae)*. *Genisto aristatae-Quercetum suberis*: Meso-Mediterranean acidophilous cork oak forest stand. *Ilici aquifoliae-Quercetum austrotyrrhenicae*: Acidophilous supra-Mediterranean forest community dominated by arborescent *Ilex aquifolium* mainly associated with *Quercus petraea* subsp. *austrotyrrhenica* and other plant species (*Acer obtusatum*, *Acer campestre*, *Ulmus glabra*). *Quercetum leptobalani*: Acidophilous meso-Mediterranean deciduous community typified by *Quercus leptobalanos* growing together with other oak species (*Q. dalechampii*, *Q. congesta*, *Quercus amplifolia*, *Q. ilex*). *Geranio versicoloris-Quercetum ilicis*: Meso-Mediterranean forest of *Q. ilex* growing on flysch at an altitude of 900-1200 m. This acidophilous plant community, is characterized by the dominance of *Q. ilex*, growing together with *I. aquifolium*. *Platano-Salicetum pedicellatae*: Thermo-Mediterranean Hyblean plateau riparian forest dominated by *Platanus orientalis* growing in association with *Salix* spp., *Populus* spp., *Fraxinus oxycarpa*, and *Nerium oleander*. *Salicetum albo—pedicellatae*: Thermo-Mediterranean riparian forest communities growing on soils with a high water table. It is characterized by *Salix* spp. and *Populus* spp. in association with *F. oxycarpa*. [e] EUNIS habitat: http://eunis.eea.europa.eu/habitats/1176. [f] CAC = *Phytophthora cactorum*; CAM = *P.* ×*cambivora* (previously *P. cambivora*); CIP = *P. citrophthora*; CIT = *Phytophthora citricola* 12; CRA = *P. crassamura*; GON = *P. gonapodyides*; KEL = *Phytophthora* sp. kelmania; LAC = *P. lacustris*; MEG = *P. megasperma*; MUL = *P. multivora*; PLU = *P. plurivora*; POL = *P. polonica*; QUE = *P. quercina*; PSC = *P. pseudocryptogea*; PSY = *P. psychrophila*; TYR = *P. tyrrhenica*; VUL = *P. vulcanica*. [g] *Pythium* sp. alternatum-like isolated. [h] *Gibberella moniliformis* also isolated. [i] *Pythium* and *Phytopythium* spp. also isolated. [j] *Elongisporangium anandrum* also isolated. [k] *Pythium* sp. JN5-like also isolated.

Table 2. Vegetation and geological features of drainage basins of 14 rivers surveyed in nine Protected Natural Areas in Sicily; location of sites with baiting rafts, and *Phytophthora* taxa isolated.

River	Protected Natural Area [a]	Location of Drainage Basin	Forest Vegetation in Drainage Basin (Natura 2000 Code, Forest Stand Type, Phytocoenosis) [b,c,d]	Geological Features of Drainage Basin	Raft No.	Municipality	Geographic Coordinates (DATUM WGS84	Altitude (m a.s.l.)	*Phytophthora* spp. [e]
Anapo	Pantalica RNR	Northern area of eastern sector of the Hyblean plateau	Natura 2000 CODE:92C0 Forest stand types: Riparian forests. Phytocoenosis: *Platano-Salicetum pedicellatae*	Limestone (algal calcarenites and calcirudites)	1	Sortino (SR)	37°07′48.0″ N–15°01′26.5″ E	294	LAC
					2	Sortino (SR)	37°07′48.0″ N–15°01′26.5″ E	294	LAC
					3	Sortino (SR)	37°08′19.3″ N–15°02′13.3″ E	219	CIP, LAC, PSC
					4	Sortino (SR)	37°08′19.3″ N–15°02′13.3″ E	219	-
Ciane	Ciane RNR	Eastern area of eastern sector of the Hyblean plateau	Natura 2000 CODE: 92A0 Forest stand types: Riparian forests. Phytocoenosis: *Salicetum albo-pedicellatae*	Alluvial sediments (derived from loam and sandy limestone)	5	Siracusa (SR)	37°02′34.4″ N–15°13′37.5″ E	4	KEL, LAC, PSC
					6	Siracusa (SR)	37°02′34.4″ N–15°13′37.5″ E	4	FRI, LAC
					7	Siracusa (SR)	37°02′34.4″ N–15°13′37.5″ E	4	LAC, MUL
					8	Siracusa (SR)	37°02′34.4″ N–15°13′37.5″ E	4	LAC, PSC
					9	Siracusa (SR)	37°02′34.4″ N–15°13′37.5″ E	4	LAC
					10	Siracusa (SR)	37°02′34.4″ N–15°13′37.5″ E	4	LAC
					11	Siracusa (SR)	37°02′34.4″ N–15°13′37.5″ E	4	LAC
					12	Siracusa (SR)	37°02′34.4″ N–15°13′37.5″ E	4	LAC
Cassibile	CavagrandeRNR	Eastern area of western sector of the Hyblean plateau	Natura 2000 CODE: 92C0 Forest stand types: Riparian forests. Phytocoenosis: *Platano-Salicetum pedicellatae*	Limestone (algal calcarenites and calcirudites)	13	Siracusa (SR)	36°57′2.05″N–15°11′11.22″E	8	HYD, LAC, PSC
					14	Siracusa (SR)	36°57′2.05″N–15°11′11.22″E	8	HYD, LAC
Irminio	Irminio SCI	Northwestern area of western sector of the Hyblean plateau	Natura 2000 CODE: 92C0 Forest stand types: Riparian forests. Phytocoenosis: *Platano-Salicetum pedicellatae*	Limestone and claystone (calcarenites and marns)	15	Ragusa (RG)	37°00′23.3″ N–14°46′45.1″ E	400	-
					16	Ragusa (RG)	37°00′23.3″ N–14°46′45.1″ E	400	-
					17	Ragusa (RG)	36°57′20.7″ N–14°46′06.2″ E	300	LAC [f]
Alcantara	Nebrodi RP	Southeastern area of Nebrodi mountains	Natura 2000 CODE:92A0 Forest stand types: Riparian forests. Phytocoenosis: *Salicetum albo-purpurae*	Numidian Flysch (quarzarenites and claystones)	18	Randazzo (CT)	37°52′50.4″ N–14°56′49.6″ E	718	GON, LAC [g]
Fiume di Troina	Nebrodi RP	Southeastern area of Nebrodi mountains	Natura 2000 CODE:91AA, 91M0, 92A0 Forest stand types: Woodlands and riparian forests. Phytocoenosis: *Erico-Quercetum virgilianae*; *Arrhenathero nebrodensis-Quercetum cerridis*; *Salicetum albo-purpureae*.	Numidian Flysch (quarzarenites and claystones)	19	San Teodoro (ME)	37°48′32.2″ N–14°41′53.1″ E	605	LAC [h]
					26	San Teodoro (ME)	37°48′32.2″ N–14°41′53.1″ E	605	LAC

Table 2. *Cont.*

River	Protected Natural Area [a]	Location of Drainage Basin	Forest Vegetation in Drainage Basin (Natura 2000 Code, Forest Stand Type, Phytocoenosis) [b,c,d]	Geological Features of Drainage Basin	Raft No.	Municipality	Geographic Coordinates (DATUM WGS84	Altitude (m a.s.l.)	*Phytophthora* spp. [e]
Flascio	Nebrodi RP	Southeastern area of Nebrodi mountains	Natura 2000 CODE:92A0 Forest stand types: Riparian forests Phytocoenosis: *Salicetum albo-purpureae*	Numidian Flysch (quarzarenites and claystones)	20	Randazzo (CT)	37°52′51.4″ N–14°52′50.6″ E	856	LAC [h,i]
					21	Randazzo (CT)	37°52′51.4″ N–14°52′50.6″ E	856	LAC [i]
Della Saracena	Nebrodi RP	Southeastern area of Nebrodi mountains	Natura 2000 CODE:91AA, 92A0 Forest stand types: Woodlands and riparian forests. Phytocoenosis: *Erico-Quercetum virgilianae*; *Salicetum albo-purpureae*.	Numidian Flysch (quarzarenites and claystones)	22	Bronte (CT)	37°52′07.3″ N–14°50′56.2″ E	811	CAM, GON, LAC, POL [i,j]
					25	Maniace (CT)	37°51′02.6″ N–14°48′04.3″ E	624	GON, LAC
Martello	Nebrodi RP	Southeastern area of Nebrodi mountains	Natura 2000 CODE:91M0, 92A0 Forest stand types: Woodlands and riparian forests. Phytocoenosis: *Arrhenathero nebrodensis-Quercetum cerridis*; *Salicetum albo-purpurae*.	Numidian Flysch (quarzarenites and claystones)	23	Maniace (CT)	37°51′27.7″ N–14°47′29.8″ E	676	LAC
Cutò	Nebrodi RP	Southern area of Nebrodi mountains	Natura 2000 CODE:92A0 Forest stand types: Riparian forests Phytocoenosis: *Salicetum albo-purpurae*	Numidian Flysch (quarzarenites and claystones)	24	Maniace (CT)	37°51′57.9″ N–14°46′00.4″ E	708	LAC
Sciambro	Etna RP	Northeastern area of Volcano Etna	Natura 2000 CODE:9530 Forest stand types: Woodland Phytocoenosis: *Junipero hemisphaericae-Pinetum calabricae*.	Volcanic (Alcali-Basalt-Na)	27	Linguaglossa (CT)	37°46′58.9″ N–15°3′04.7″ E	1656	GON
					28	Linguaglossa (CT)	37°46′58.4″ N–15°3′02.5″ E	1669	-
					29	Linguaglossa (CT)	37°46′57.0″ N–15°2′01.8″ E	1682	GON
Fiumefreddo	FiumefreddoRNR	Northeastern boundary of Volcano Etna	Natura 2000 CODE:92A0 Forest stand types: Riparian Forests Phytocoenosis: *Populetalia albae* The following were also present: (i) citrus groves, (ii) nurseries; (iii) artificial forest of *Eucalyptus* and *Carya cordiformis*.	Alluvial sediments (derived from loam, sandy limestone, and volcanic rocks).	30	Fiumefreddo di Sicilia (CT)	37°47′22.15″ N–15°13′55.63″ E	6	LAC, MUL, PLU, PSC, THE
					31	Fiumefreddo di Sicilia (CT)	37°47′25.98″ N–15°14′3.89″ E	6	LAC, PSC, THE

Table 2. *Cont.*

River	Protected Natural Area [a]	Location of Drainage Basin	Forest Vegetation in Drainage Basin (Natura 2000 Code, Forest Stand Type, Phytocoenosis) [b,c,d]	Geological Features of Drainage Basin	Raft No.	Municipality	Geographic Coordinates (DATUM WGS84	Altitude (m a.s.l.)	*Phytophthora* spp. [e]
Fiumara d'Agrò	Agrò SCI	Southeastern area of Peloritani mountains	Natura 2000 CODE:91AA, 91E0 Forest stand types: Woodlands and Riparian Forests. Phytocoenosis: *Erico-Quercetum virgilianae*; *Spartio-Nerietum oleandri*.	Metamorphic (Phyllites)	32	Limina (ME)	37°57′22.4″ N–15°16′20.8″ E	202	LAC, PLU, PSC
					33	Limina (ME)	37°57′22.4″ N–15°16′20.8″ E	202	-
Fiumedinisi	Fiumedinisi RNR	Southeastern area of Peloritani mountains	Natura 2000 CODE:91AA, 92A0; 92C0. Forest stand types: Woodlands and Riparian Forests. Phytocoenosis: *Erico-Quercetum virgilianae*; *Platano-Salicetum gussonei*; *Salicetum albo-purpuree*.	Metamorphic (mainly green shists and amphibolites)	34	Fiumedinisi (ME)	38°01′47.8″ N–15°22′21.3″ E	214	-
					35	Fiumedinisi (ME)	38°01′47.8″ N–15°22′21.3″ E	214	CIP, LAC [g]

[a] Etna RP = Etna Regional Park; Nebrodi RP = Nebrodi Regional Park; Madonie RP = Madonie Regional Park; Pantalica RNR = Pantalica, Valle dell'Anapo e Torrente Cavagrande Regional Natural Reserve (RNR); Ciane RNR = Fiume Ciane e Saline di Siracusa RNR; Cavagrande RNR = Cavagrande del Cassibile RNR; Fiumedinisi RNR = Fiume Fiumedinisi e Monte Scuderi RNR; Agrò SCI = ITA030019—Tratto Montano del Bacino della Fiumara di Agrò—Site of Community Importance (SCI); Irminio SCI = ITA080002—Alto corso del Fiume Irminio SCI. [b] Forest vegetation features were in accordance with Natura 2000 sites data and respective management plans: ftp://ftp.minambiente.it/PNM/Natura2000/TrasmissioneCE_2016/schede_mappe/Sicilia/SIC_schede/. [c] Natura 2000 Habitats: http://www.minambiente.it/sites/default/files/archivio/allegati/rete_natura_2000/int_manual_eu28.pdf [d] *Platano-Salicetum pedicellatae*: Thermo-Mediterranean Hyblean plateau riparian forest dominated by *Platanus orientalis* growing in association with *Salix* spp., *Populus* spp., *Fraxinus oxycarpa*, and *Nerium oleander*. *Salicetum albo-pedicellatae*: Thermo-Mediterranean riparian forest communities that grow on soils with a high water table. It is characterized by *Salix* spp. and *Populus* spp. in association with *F. oxycarpa*. *Salicetum albo-purpurea*: thermo-meso-Mediterranean riparian forest dominated by *Salix purpurea*, *Salixalba*, and *Salix pedicellata* in association with *Populus* spp. *Arrhenathero nebrodensis-Quercetum cerridis*: meso-Mediterranean acidophilous turkey oak forest stand typified by *Quercus cerris*; at higher altitudes (ca. 1400 m) it is mixed with beech forests (*Anemono apenninae-Fagetum sylvaticae*). *Populetalia albae*: riparian forests characterized by communities of *S. alba* and *Populus alba*. *Erico-Quercetum virgilianae*: meso-Mediterranean acidophilous woodland dominated by *Quercus dalechampii* in association with *Fraxinus ornus*. *Spartio-Nerietum oleandri*: thermo-Mediterranean community characteristic of Sicilian "Fiumara" streams, dominated by *N. oleander* in association with *Salix* spp. and *Populus* spp. *Platano-Salicetum gussonei*: thermo-Mediterranean community characteristic of Sicilian "Fiumara" streams, typified by *P. orientalis* and *Salix gussonei*. *Junipero hemisphaericae-Pinetum calabricae*: supra-Mediterranean Calabrian laricio pine forest with a dense structure. [e] CAM = *Phytophthora ×cambivora*; CIP = *P. citrophthora*; FRI = *P. frigida*, GON = *P. gonapodyides*; HYD = *P. hydropathica*; KEL = *P.* sp. kelmania; LAC = *P. lacustris*; MUL = *P. multivora*; PLU = *P. plurivora*; POL = *P. polonica*, PSC = *P. pseudocryptogea*; THE = *P. thermophila*. [f] *Pythium* sp. JN6-like also isolated. [g] *Pythium* sp. strain 1-9-like also isolated. [h] *Pythium* sp. F-1509-like also isolated. [i] *Pythium* sp. dissotocum-like also isolated. [j] *Pythium* sp. FL-2016d-like also isolated.

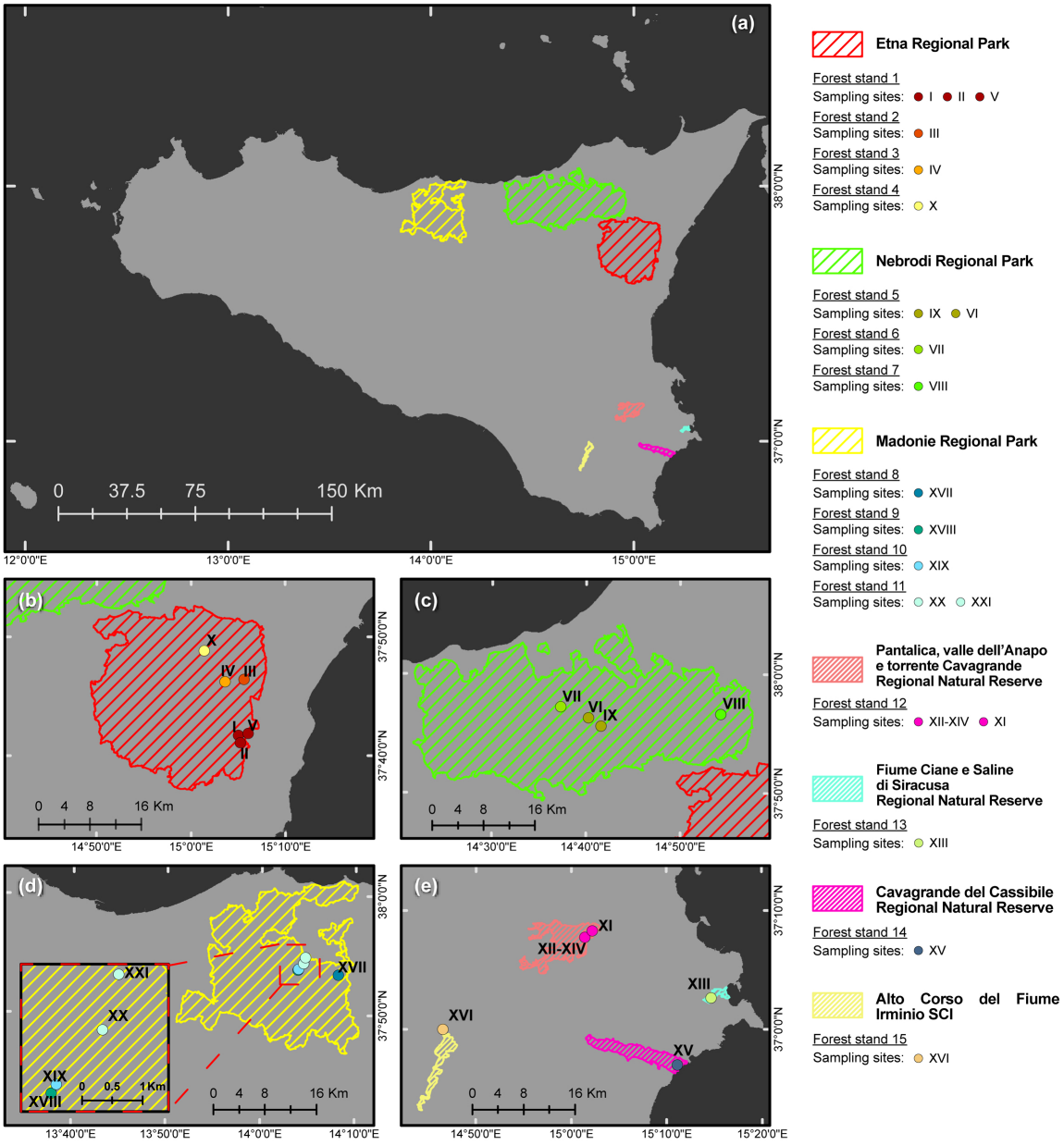


Figure 1. Geographical location of the 15 forest stands and the seven Protected Natural Areas included in the *Phytophthora* survey of natural forests in Sicily, projected using the Universal Transverse Mercator (UTM) (**a**). Location of the sampled forest sites within the Etna (**b**), Nebrodi (**c**), and Madonie (**d**) Regional Parks; and in the "Pantalica, valle dell'Anapo e torrente Cavagrande", "Fiume Ciane e Saline di Siracusa", "Cavagrande del Cassibile" Regional Natural Reserves and the "ITA080002—Alto Corso del Fiume Irminio" Site of Community Importance (SCI) (**e**).

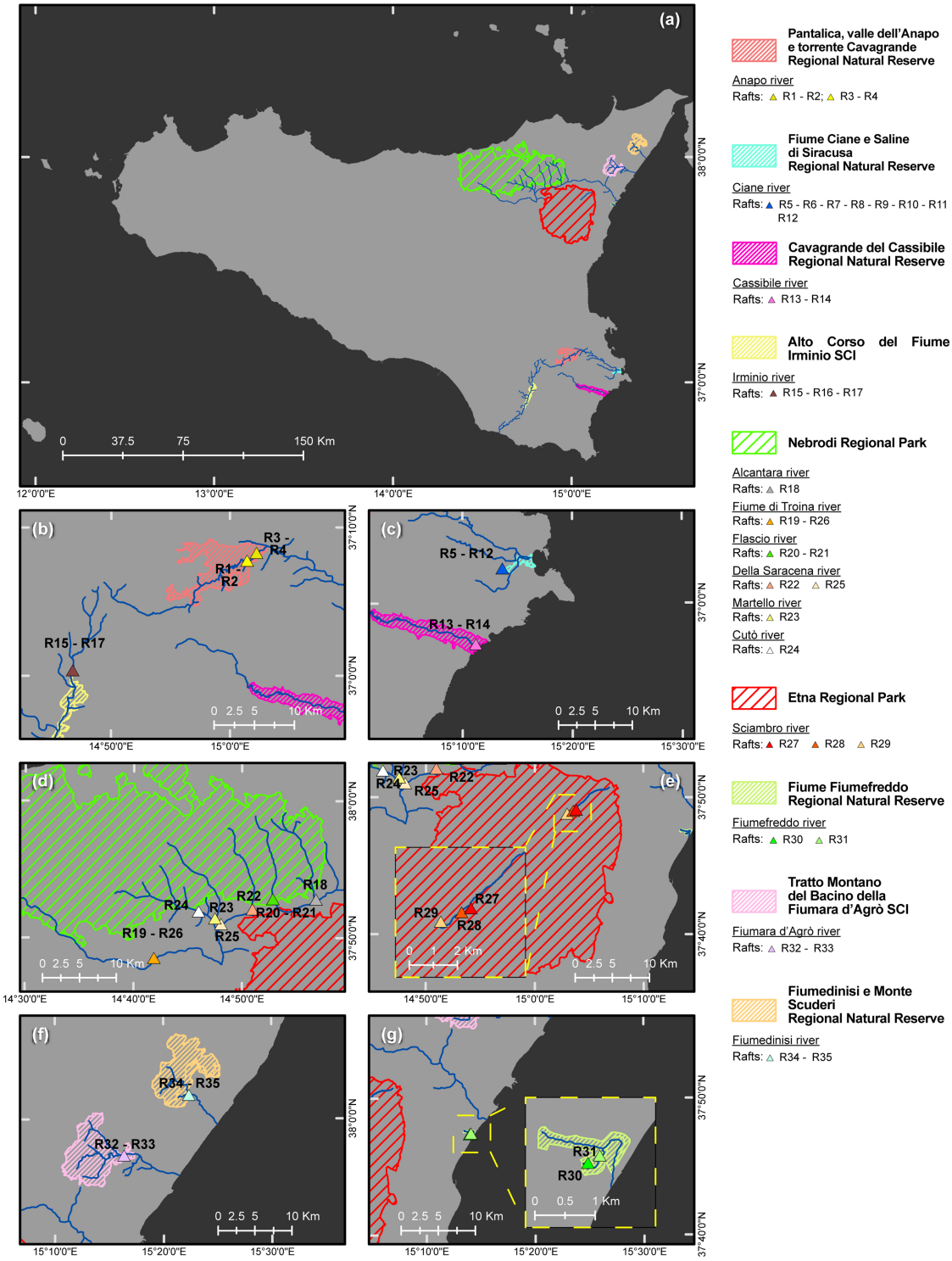


Figure 2. Geographical location of the nine Protected Natural Areas and the 14 river systems included in the *Phytophthora* survey of rivers in Sicily, projected using the Universal Transverse Mercator (UTM) (**a**). Riparian sampling sites (R) along the river systems running through: "Pantalica, valle dell'Anapo e Torrente Cavagrande" Regional Natural Reserve (RNR) and "ITA080002—Alto Corso del Fiume Irminio" Site of Community Importance (SCI) (**b**); "Fiume Ciane e Saline di Siracusa" and "Cavagrande del Cassibile" RNRs (**c**); "Nebrodi" (**d**) and "Etna" (**e**) Regional Parks; "ITA030019—Tratto Montano del Bacino della Fiumara d'Agrò" SCI and "Fiumedinisi e Monte Scuderi" RNR (**f**); and "Fiume Fiumefreddo" RNR (**g**).

2.2. Morphological Characterization of Isolates

Seven-days-old cultures grown at 20 °C in the dark on V8A were used to group all obtained isolates into morphotypes on the basis of their colony growth patterns. In addition, morphological features of sporangia, oogonia, antheridia, chlamydospores, hyphal swellings, and aggregations were examined [20,42] and compared with species descriptions in the literature.

2.3. Molecular Identification of Isolates

Molecular analyses were performed with 387 (184 from soil and 203 from rivers) of the 841 obtained isolates, representative of all morphotypes, soil samples, and baiting rafts. DNA was extracted from pure cultures grown on V8A using the PowerPlant® Pro DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's protocol. DNA was stored at −20 °C until further use.

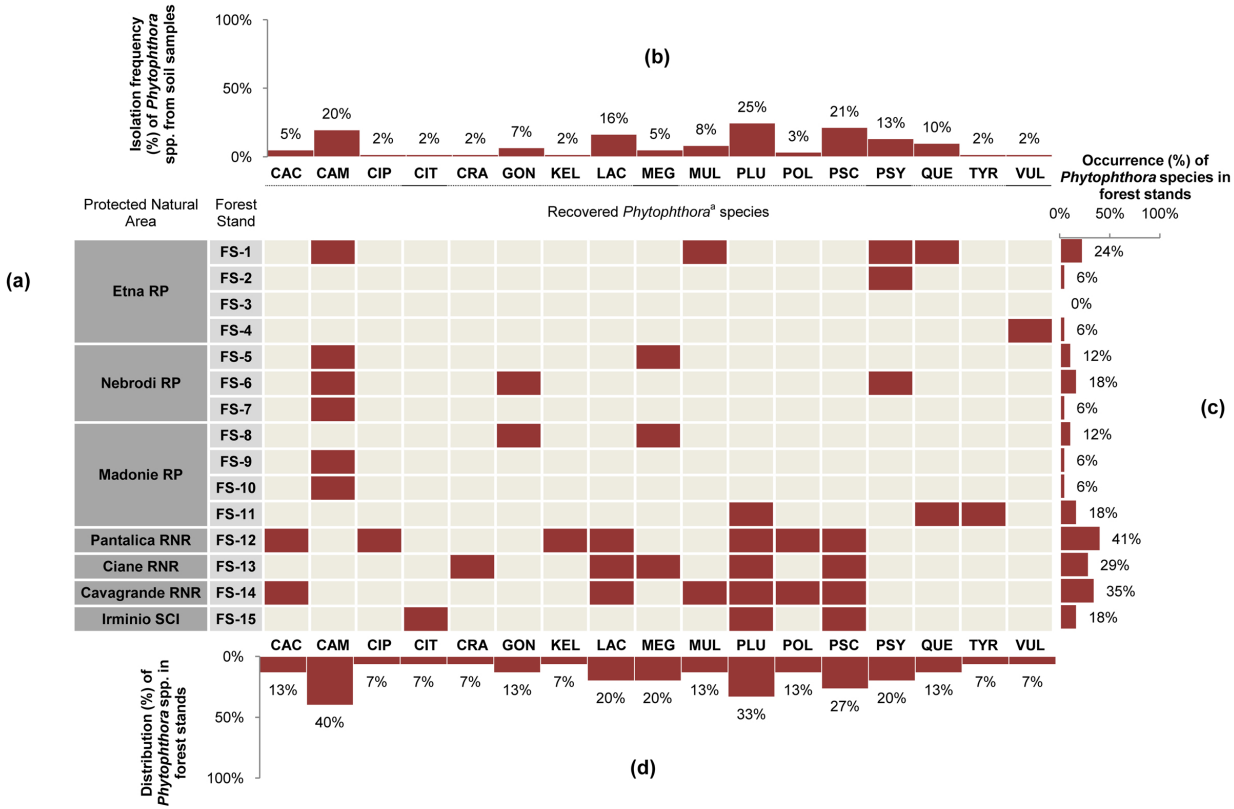
The identification of *Phytophthora* species was performed by sequence analysis of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). For amplification, forward primers ITS6 or ITS1 [44] and reverse primer ITS4 were used [45]. The PCR amplification mix and thermocycler conditions were as in [44]. PCR products were purified and sequenced by Macrogen Europe (Amsterdam, The Netherlands) in both directions with the primers used for amplification. Sequences were analyzed using FinchTV v.1.4.0 (https://digitalworldbiology.com/FinchTV). For species identification, blast searches in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and in a local database containing sequences of ex-type or key isolates from published studies were performed. Isolates were assigned to a species when their sequences were at least 99% identical to a reference isolate. ITS sequences from representative isolates of this study were deposited at GenBank (www.ncbi.nlm.nih.gov/genbank; accession numbers are given in Table S2).

3. Results

Morphological and ITS sequence analyses revealed the occurrence of multiple *Phytophthora* species in each of the sampled PNAs. ITS sequence analyses showed that 351 of the 387 (90.7%) analyzed isolates (162 from forest soils and 189 from rivers) matched with 99–100% identity reference sequences of 16 known *Phytophthora* species and the designated *Phytophthora* sp. kelmania [46]. Nine isolates belonged to two species recently described as *Phytophthora vulcanica* and *Phytophthora tyrrhenica* [6] from Clade 7a, and to a new, yet undescribed species from Clade 2, while 27 isolates (7.0%) were assigned to other oomycete genera (Tables 1 and 2).

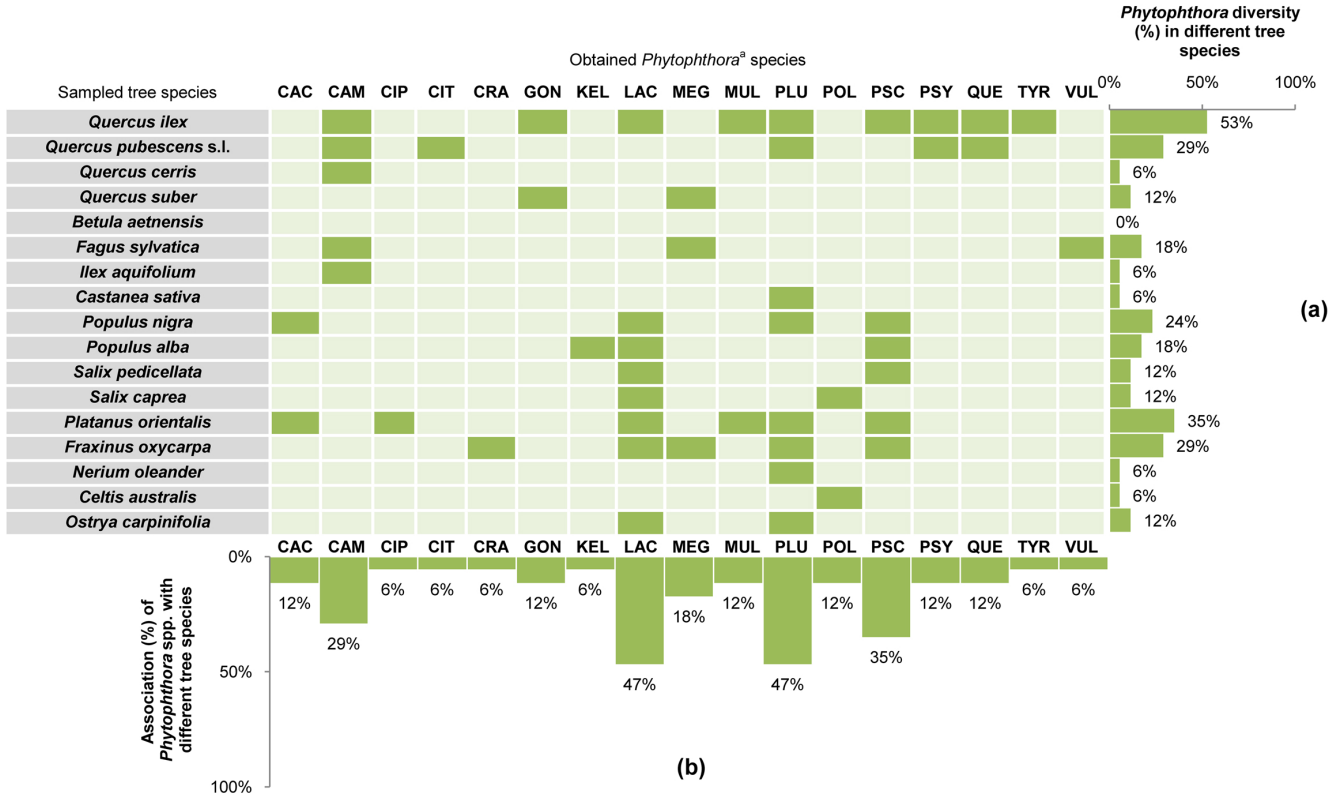
3.1. Phytophthora Diversity and Distribution in Forest Stands

In all oak and beech forests sampled, the majority of trees showed disease symptoms including thinning and dieback of crowns, fine root losses and, in some cases, bleeding stem cankers, whereas in riparian forests diseased trees had a scattered distribution. Noteworthy, in the riparian forest FS-13 along the Ciane river, is the fact that almost all *Fraxinus oxycarpa* Bieb. trees showed severe dieback and mortality. Overall, in all seven selected PNAs, *Phytophthora* species were found in 14 of 15 sampled forest stands (93.3%). In total, 17 *Phytophthora* species from eight of the 12 known phylogenetic clades [6] were isolated from 61 of the 83 (73.5%) soil samples collected from 16 of the 17 tree species sampled (94%) (Table 1, Figure 3a,c, Figure 4a,b, and Figure S2a,c,e). Only in one forest stand (FS-3) could no *Phytophthora* isolates be obtained from the only tested tree species *Betula aetnensis* Raf.



CAC = *Phytophthora cactorum*; CAM = *Phytophthora ×cambivora*; CIP = *Phytophthora citrophthora*; CIT = *Phytophthora citricola* 12; CRA = *Phytophthora crassamura*; GON = *Phytophthora gonapodyides*; KEL = *Phytophthora* sp. kelmania; LAC = *Phytophthora lacustris*; MEG = *Phytophthora megasperma*; MUL = *Phytophthora multivora*; PLU = *Phytophthora plurivora*; POL = *Phytophthora polonica*; PSC = *Phytophthora pseudocryptogea*; PSY = *Phytophthora psychrophila*; QUE = *Phytophthora quercina*; TYR = *Phytophthora tyrrhenica*; VUL = *Phytophthora vulcanica*.

Figure 3. Distribution and diversity of *Phytophthora* species in sampled forest stands from Protected Natural Areas in Sicily. (**a**) Etna RP = Etna Regional Park; Nebrodi RP = Nebrodi Regional Park; Madonie RP = Madonie Regional Park; Pantalica RNR = Pantalica, Valle dell'Anapo e Torrente Cavagrande Regional Natural Reserve (RNR); Ciane RNR = Fiume Ciane e Saline di Siracusa RNR; Cavagrande RNR = Cavagrande del Cassibile RNR; Irminio SCI = ITA080002—Alto corso del Fiume Irminio Site of Community Importance (SCI), (**b**) isolation frequency (%) of *Phytophthora* species from *Phytophthora*-positive soil samples, (**c**) occurrence (%) of *Phytophthora* species in sampled forest stands, (**d**) distribution (%) of *Phytophthora* species in the sampled forest stands.



CAC = *Phytophthora cactorum*; CAM = *Phytophthora ×cambivora*; CIP = *Phytophthora citrophthora*; CIT = *Phytophthora citricola* 12; CRA = *Phytophthora crassamura*; GON = *Phytophthora gonapodyides*; KEL = *Phytophthora* sp. kelmania; LAC = *Phytophthora lacustris*; MEG = *Phytophthora megasperma*; MUL = *Phytophthora multivora*; PLU = *Phytophthora plurivora*; POL = *Phytophthora polonica*; PSC = *Phytophthora pseudocryptogea*; PSY = *Phytophthora psychrophila*; QUE = *Phytophthora quercina*; THY = *Phytophthora tyrrhenica*; VUL = *Phytophthora vulcanica*.

Figure 4. Association of *Phytophthora* species with different tree species in Protected Natural Areas in Sicily. Dark-green color represents a *Phytophthora*—host tree association, (**a**) diversity of *Phytophthora* species in different tree species (in % of all *Phytophthora* species found), (**b**) association of *Phytophthora* species with the sampled tree species (in % of all tree species sampled).

Species from Clade 7, i.e., *Phytophthora ×cambivora* (previously *P. cambivora*), *Phytophthora vulcanica*, and *Phytophthora tyrrhenica*, were isolated from 53% of the sampled forest stands (Figure 3 and Figure S2b) in three of the seven protected natural areas (Etna, Nebrodi, and Madonie RPs) (Table 1, Figure 3a, and Figure S2a,d). *Phytophthora ×cambivora* was isolated from all sampled meso-, and supra-Mediterranean forest stands: In the Etna RP (FS-1) from *Quercus pubescens* Willd. *sensu latu* (s.l.); in the Nebrodi RP from *Fagus sylvatica* L., *Quercus cerris* L., *Quercus ilex* L., and *Q. pubescens* s.l. and all sampled forest stands (FS-5 to FS-7); and in the Madonie RP (FS-9, FS-10) from *Ilex aquifolium* L. and *Q. pubescens* s.l. (Table 1, Figure 3a,b,d, and Figure 4). *Phytophthora ×cambivora* occurred in an altitude range between 660 and 1780 m above sea level (a.s.l.). *Phytophthora vulcanica* and *P. tyrrhenica* were recovered from *F. sylvatica* in FS-4 and from *Q. ilex* in FS-11, respectively (Table 1).

Four Clade 6 species, *Phytophthora gonapodyides*, *Phytophthora megasperma*, *Phytophthora lacustris*, and *Phytophthora crassamura*, were found in 40% of the sampled forest stands in five PNAs (Figure 3 and Figure S2b). *Phytophthora gonapodyides* occurred between 700 and 1000 m a.s.l. in the rhizosphere of *Q. ilex* and *Quercus suber* L. in meso-Mediterranean evergreen (FS-6) and cork oak (FS-8) woodlands, respectively (Table 1, Figure 4). *Phytophthora megasperma* was isolated from supra-, meso-, and thermo-Mediterranean forest stands in three PNAs: In the Nebrodi RP from *F. sylvatica* (FS-5); in the Madonie RP from *Q. suber* (FS-8); and in the Fiume Ciane e Saline di Siracusa RNR (Ciane RNR) from *Fraxinus oxycarpa* (FS-13). This *Phytophthora* species inhabited a wide altitudinal range between 4 and 1450 m a.s.l. (Table 1, Figure 3a,b,d, and Figure 4). *Phytophthora lacustris* was isolated from the rhizosphere of eight different tree species between 4 and 236 m a.s.l. in three thermo-Mediterranean riparian forest stands (FS-12 to FS-14) located in three PNAs (Table 1, Figure 3a,b,d, and Figure 4). *Phytophthora crassamura* only occurred in the rhizosphere of *F. oxycarpa* in the Ciane RNR (FS-13) (Table 1, Figure 4).

Species from *Phytophthora* Clade 2 were present in six of the seven monitored PNAs (Figure S2a,b,d). *Phytophthora plurivora* was most widespread, occurring in 25% of the *Phytophthora*-positive soil samples taken from eight different tree species in 33% of the sampled forest stands (FS-11 to FS-15) and in five PNAs (Table 1, Figure 3a,b,d, and Figure 4). Interestingly, this pathogen was recovered from seven trees species in the riparian thermo-Mediterranean plane tree stand (FS-12) of the Pantalica RNR. The altitudinal distribution of *P. plurivora* ranged from an altitude of 4 to 850 m a.s.l. *Phytophthora multivora* was associated with *Q. ilex* in the Etna RP (FS-1) and with *Platanus orientalis* L. in the Cassibile RNR (FS-14) (Table 1, Figure 3a,b,d). *Phytophthora citrophthora* was only found in the rhizosphere of *P. orientalis* in FS-12 (Table 1). A previously unknown species from the '*Phytophthora citricola* complex', informally designated here as *P. citricola* 12, was recovered from the rhizosphere of *Q. pubescens* s.l. in riparian stand FS-15 in Irminio SCI (Table 1).

The Clade 1 species *Phytophtora cactorum* occurred in two riparian thermo-Mediterranean plane tree forests in two PNAs (Table 1, Figure S2a,b,d, and Figure 3a,b,d). In the Pantalica RNR (FS-12) and the Cassibile RNR (FS-14), *P. cactorum* was associated with *P. orientalis* and *Populus nigra* L., respectively (Figure 4).

The Clade 3 species *Phytophthora psychrophila* was found associated with *Q. pubescens* s.l. in two forest stands (FS-1, FS-2) of the Etna RP and with *Q. ilex* in stand FS-6 of the Nebrodi RP. The altitudinal distribution ranged from 890 to 1345 m a.s.l. (Table 1).

Phytophthora pseudocryptogea from Clade 8 was frequently isolated at an altitude between 4 and 240 m from rhizosphere soil of six different tree species in four riparian thermo-Mediterranean forest stands (FS-12 to FS-15) located in four distinct PNAs (Table 1, Figure 3a,b,d). Another Clade 8 taxon, *Phytophthora* sp. kelmania, was detected in only one soil sample from *Populus alba* L. in stand FS-12 (Table 1, Figure 4).

Phytophthora polonica from Clade 9 was associated with *Celtis australis* L. and *Salix caprea* L. in two riparian thermo-Mediterranean forest stands, FS-12 in the Pantalica RNR and FS-14 in the Cavagrande RNR, respectively (Table 1, Figure 3a,b,d, and Figure 4).

The oak-specific pathogen *Phytophthora quercina* from the recently described Clade 12 [6] was recovered between 660 and 1110 m a.s.l. from *Q. ilex* and *Q. pubescens* s.l. at two sites of FS-1 in the Etna RP and from *Q. ilex* in the Madonie RP (FS-11) (Table 1, Figure 3a,b,d and Figure 4).

3.2. Phytophthora Diversity and Distribution in Rivers within PNAs

In total 12 *Phytophthora* species from five phylogenetic clades were detected in all monitored rivers running through all nine selected PNAs (Table 2, Figure 5a,c, and Figure S3a–f); 29 of the 35 baiting rafts (83%) were *Phytophthora*-positive.

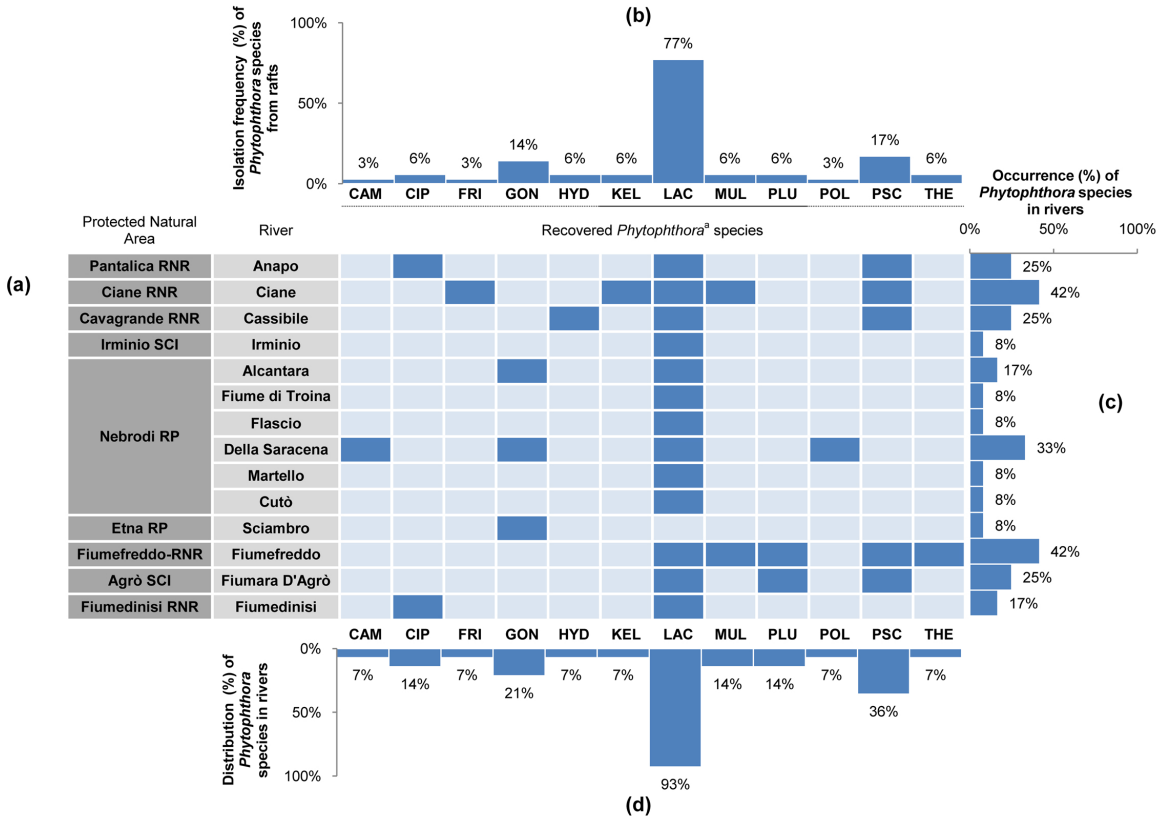
Most common were mainly aquatic *Phytophthora* species from ITS Clade 6 that were recovered from all monitored river systems and PNAs (Figure S3a,d,e). *Phytophthora lacustris* occurred between 4 and 850 m a.s.l. in 77% of the baiting rafts and in all watercourses except for the Sciambro river (Table 2, Figure 5a,b,d), a torrential high altitude stream, which only flows seasonally during snowmelt. In five rivers, *P. lacustris* was the only *Phytophthora* species detected. *Phytophthora gonapodyides* was found in an altitudinal range between ca. 700 and 1700 m a.s.l. in the Alcantara and Della Saracena rivers (Nebrodi RP) and in the Sciambro river (Etna RP); in the latter it was the only *Phytophthora* species isolated (Table 2, Figure 5a,b,d). The third mainly aquatic Clade 6 species, *Phytophthora thermophila*, was exclusively found in the Fiumefreddo river (Table 2, Figure 5a,b,d).

The Clade 2 species, *P. plurivora*, *P. multivora*, *P. citrophthora*, and *P. frigida*, were isolated from 36% of the rivers in five PNAs at lowland sites ranging from 6 to 220 m a.s.l. (Table 2, Figure 5a,b,d and Figure S3a,d,e). While *P. frigida* was only found in Ciane river, each of the other species of Clade 2 occurred in two rivers: *P. plurivora* in the Fiumara d'Agrò and Fiumefreddo rivers, *P. multivora* in the Fiumefreddo and Ciane rivers, and *P. citrophthora* in the Anapo and Fiumedinisi rivers (Table 2, Figure 5).

Phytophthora ×*cambivora* from Clade 7 and *P. polonica* from Clade 9 were both exclusively detected in the Della Saracena river in the Nebrodi RP (Table 2, Figure 5).

Two species from Clade 8 were found in five watercourses running through five PNAs (Table 2, Figure S3a,d,e). *Phytophthora pseudocryptogea* was widespread, occurring between 4 and 220 m a.s.l. in the Anapo, Ciane, Cassibile, Fiumefreddo, and Fiumara d'Agrò rivers, whereas *P.* sp. *kelmania* was exclusively isolated from the Ciane river (Table 2, Figure 5a,b,d).

Phytophthora hydropathica from Clade 9 was only found in the Cassibile river (Table 2, Figure 5a,b,d).



CAM = *Phytophthora* ×*cambivora*; CIP = *Phytophthora citrophthora*; FRI = *Phytophthora frigida*; GON = *Phytophthora gonapodyides*; HYD = *Phytophthora hydropatica*; KEL = *Phytophthora* sp. kelmania; LAC = *Phytophthora lacustris*; MUL = *Phytophthora multivora*; PLU = *Phytophthora plurivora*; POL = *Phytophthora polonica*; PSC = *Phytophthora pseudocryptogea*; THE = *Phytophthora thermophila*.

Figure 5. Distribution and diversity of *Phytophthora* species in sampled rivers from Protected Natural Areas in Sicily. (**a**) Etna RP = Etna Regional Park; Nebrodi RP = Nebrodi Regional Park; Madonie RP = Madonie Regional Park; Pantalica RNR = Pantalica, Valle dell'Anapo e Torrente Cavagrande Regional Natural Reserve (RNR); Ciane RNR = Fiume Ciane e Saline di Siracusa RNR; Cavagrande RNR = Cavagrande del Cassibile RNR; Fiumedinisi RNR = Fiume Fiumedinisi e Monte Scuderi RNR; Agrò SCI = ITA030019—Tratto Montano del Bacino della Fiumara di Agrò—Site of Community Importance (SCI); Irminio SCI = ITA080002—Alto corso del Fiume Irminio SCI, (**b**) isolation frequency (%) of *Phytophthora* species from *Phytophthora*-positive baiting rafts, (**c**) occurrence (%) of *Phytophthora* species in sampled rivers, (**d**) distribution (%) of *Phytophthora* species in the sampled rivers.

4. Discussion

This is the first study of *Phytophthora* diversity in Europe using conventional isolation methods and covering both a wide range of natural forest types and watercourses crossing these areas. Previously, the only surveys of *Phytophthora* diversity in both forests and rivers within the same region in Europe used only a metabarcoding approach which is based on DNA identification technologies and high-throughput DNA sequencing. In Spain, 13 and 35 *Phytophthora* phylotypes were detected in forest soils and streams, respectively [37]. Using a different molecular method, a survey in Scotland demonstrated the presence of 10 and 9 *Phytophthora* phylotypes in soil and water samples, respectively [47]. The present survey unveiled a rich community of 20 *Phytophthora* species in the Sicilian PNAs studied. With 17 different species from 8 of the 12 known phylogenetic clades, including the two newly described species *P. tyrrhenica* and *P. vulcanica* [6], *Phytophthora* diversity in 15 natural forest stands was higher than in previous broadleaved forest surveys in Europe using similar isolation methods. In oak forests across Italy, northeastern France, Austria, and Turkey, and in oak and beech forests in Bavaria 11, 8, 5, 7, and 13 *Phytophthora* species, respectively, were found [9,13,17,48,49]. However, the lower *Phytophthora* diversity in these surveys may partly be due to the limited number of tree species and forest types included. With nine *Phytophthora* species from four phylogenetic clades the diversity found in 14 rivers in Sicily was almost as high as in previous surveys in Australia, the USA, and South Africa which covered much larger areas and a higher numbers of rivers [40,50–52], but lower than in Taiwan where four described *Phytophthora* species and 14 previously unknown *Phytophthora* taxa were discovered in 19 rivers [21].

The high diversity of *Phytophthora* species in natural forests and rivers in Sicily is particularly impressive considering the relatively small area of less than 10,000 km^2 covered by this survey. This may be explained by the diversity of forest types and altitudinal zones surveyed and Sicily's long and changing history of human colonization and the introduction of non-native horticultural plants. Thirteen of the 20 *Phytophthora* species occurring in the sampled Sicilian ecosystems are considered introduced pathogens: *P. cactorum*, *P. ×cambivora*, *P. citricola* 12, *P. citrophthora*, *P. crassamura*, *P. frigida*, *P. hydropathica*, *P. multivora*, *P. plurivora*, *P. polonica*, *P. pseudocryptogea*, *P. thermophila*, and *P.* sp. kelmania [21,25,32,42,53,54]. In contrast, *P. psychrophila*, *P. quercina*, *P. tyrrhenica*, and *P. vulcanica* are considered endemic to Europe resulting from species radiation following adaptation to different Fagaceae species [6].

Amongst the 17 *Phytophthora* species obtained from forest stands, *P. ×cambivora*, *P. plurivora*, and *P. pseudocryptogea* were the most widespread whereas the other species had a more scattered or even punctual distribution. The allopolyploid hybrid pathogen *P. ×cambivora* was most common, occurring in the majority of meso- and supra-Mediterranean forest stands sampled in the Nebrodi, Etna, and Madonie Regional Parks. In a previous study, *P. ×cambivora* was also found in Corleone near Palermo [49]. Although the recovery from *I. aquifolium* extended the known host range of *P. ×cambivora*, this pathogen was mainly associated with known susceptible host species like *Quercus* spp. and *F. sylvatica* [9,13,42,49]. In most cases, oak and beech trees showed typical disease symptoms like thinning and dieback of crowns, fine root losses, and in some cases bleeding stem cankers, all indicative of *Phytophthora* infections. Due to the high aggressiveness of *P. ×cambivora* to oaks and beech [6,11,42,55] it seems likely that this pathogen is associated with the widespread decline and dieback of oak and beech stands recently reported in Sicily [5]. The results of this work confirm previous studies in Germany and Italy demonstrating that *P. ×cambivora* preferentially occurs in acidic and clayey soils [9,11,13,49,56]. Of note, *P. ×cambivora* was not isolated from riparian thermo-Mediterranean forests in Sicily. Compared to *P. ×cambivora*, *P. plurivora* showed an opposite distribution pattern, being the most common species in riparian thermo-Mediterranean forest stands dominated by willows, poplars, plane, and ash trees. However, it was only infrequently isolated from seasonally dry, meso- and supra-Mediterranean forests. This distribution is most likely caused by the thin oospore walls which make *P. plurivora* susceptible to droughts [53]. Although *P. plurivora* was already reported from more than 80 woody host species including *Castanea sativa* Mill., *F. sylvatica*, *Fraxinus* spp., *Quercus* spp.,

and *Salix* spp. [10,11,13,14,21,32,53,57–61], the recoveries from rhizosphere soil of *P. nigra*, *P. orientalis*, *Nerium oleander* L., and *Ostrya carpinifolia* Scop. in the present study constituted first-time records for this wide host range pathogen. Interestingly, *P. plurivora* showed a similar upper limit of vertical distribution as in the Bavarian Alps (ca. 870 m a.s.l.) [53]. Despite being an aggressive beech pathogen across Europe and in the USA [9,14,32,60,62,63], *P. plurivora* did not occur in the rhizosphere of *F. sylvatica* forests in Sicily, which at this southern latitude grow at altitudes above ca. 1400 m a.s.l. However, in contrast to Bavaria, in Sicily this vertical limit is most likely caused by extremely dry summers, causing desiccation of the thin-walled oospores [53], rather than by deep winter temperatures. In Taiwan, *P. plurivora* occurs at altitudes around 2000 m in regions with mild winters and humid summers [21]. *Phytophthora multivora*, the second species from the '*Phytophthora citricola* complex' found in this survey, was less common than *P. plurivora*, being isolated only from *Q. ilex* and *P. orientalis* in each one of the meso-Mediterranean evergreen oak and riparian thermo-Mediterranean forest stand, respectively, and in the Ciane and Fiumefreddo rivers. Due to its particularly thick oospore walls, *P. multivora* has adapted perfectly to severe summer droughts in Mediterranean regions such as Western Australia and South Africa, where it is widespread in both native vegetation and urban environments [51,64–66]. In Europe, *P. multivora* was recently introduced and is currently spreading through the nursery sector and in young plantings [32,67]. Prior to this study, it had only been occasionally recovered from the wider environment [60,68]. Hitherto, *P. frigida* from Clade 2 was only known from *Eucalyptus* plantations in South Africa and from rainforests in eastern Australia [54,69]. The number of known species of Clade 2 is rapidly increasing; besides *P. plurivora* and *P. multivora* it includes numerous other aggressive *Phytophthora* species. Many Clade 2 species pose serious threats to natural ecosystems across the world [10,43,53,70,71]. The findings of *P. frigida* and the new species *P. citricola* 12, and the widespread occurrence of *P. plurivora* and *P. multivora* in Sicilian PNAs are of serious concern.

In the present study, *P. pseudocryptogea* from Clade 8 was frequently recovered from six tree species in riparian thermo-Mediterranean forest stands and from five rivers. It is the first report of this species in Sicily. While *P. pseudocryptogea* was previously not reported from Sicily, its close relative *P. cryptogea* commonly causes damage to several non-native ornamentals in nurseries and tomato crops under plastic-houses [72–76]. *Phytophthora cryptogea* has a scattered, but widespread, distribution in periodically dry Mediterranean natural ecosystems [25,56,58]. In Europe, *P. cryptogea* is an established exotic pathogen, whereas *P. pseudocryptogea* and the phylogenetically close taxon *P.* sp. kelmania [46], are considered as recently introduced emerging pathogens [32].

In accord with previous studies in other areas of the world [38,39,41,50], Clade 6 species prevailed in rivers, indicating their adaptation to aquatic environments. Interestingly, two mainly aquatic opportunistic pathogens from Clade 6, *P. gonapodyides* and *P. lacustris*, which often co-occur in river systems in temperate regions of North America, Europe, and Asia [40,77–79], showed opposite distribution patterns in Sicily. *Phytophthora gonapodyides* occurred exclusively at altitudes above 620 m, where it was mainly associated with meso-Mediterranean oak stands on acidic non-calcareous soils and with rivers running through oak stands. In previous studies, *P. gonapodyides* was also often found in oak stands and on acidic sites [11,13,49,77]. In contrast, in this study, *P. lacustris* was only isolated below 850 m altitude from *Salix*-dominated riparian forests on both silica-rich acidic and calcareous alkaline sites, and from rivers running through these forests. Both *Phytophthora* species co-occurred only in three rivers in a transition zone between 624 and 811 m a.s.l. The different altitudinal preferences of both species reflect their different cardinal temperatures for growth [79]. In this survey, two other species, *P. hydropathica* and *P. thermophila* from Clade 9, were exclusively detected in rivers confirming their mainly aquatic lifestyle. Prior to the present study, *P. hydropathica* was found in rivers and irrigation reservoirs in the Eastern USA [80–82] and in rivers in Galicia, Spain [83]. In Italy, this species was only reported from ornamental plants in commercial nurseries [84]. *Phytophthora thermophila* was previously exclusively detected in streams and native forests of *Eucalyptus* and *Banksia* spp. in Australia [20] and, hence, the finding in the Fiumefreddo river constitutes the first-time report for

Europe. The presence of both a nursery and a young *Eucalyptus* plantation close to the Fiumefreddo River suggests an introduction via infested nursery plants.

Phytophthora quercina is commonly occurring across Europe, causing chronic fine root losses in different oak species which, in interaction with climatic extremes and secondary pests and pathogens, lead to decline, dieback, and mortality of oak forests [10,13,14,17,32,49,85–87]. The present findings in Sicily extend the known distribution of this pathogen to the southern oak stands of Europe.

Two previously unknown *Phytophthora* species, which have been recently described as *P. vulcanica* and *P. tyrrhenica*, were isolated from a beech stand on Mount Etna and a *Q. ilex* stand in the Madonie mountains, respectively. In a multigene phylogenetic study, both species were placed in Clade 7, closely related to *P. uliginosa*, a cryptic species which seems to be restricted to Europe [6,55]. *Phytophthora tyrrhenica* was also detected in oak stands in Sardinia [6] whereas *P. vulcanica* was recovered in Sicily for the first time. Since decline symptoms in the infested stands were only mild and both species showed limited aggressiveness to their respective host species in pathogenicity tests; they are considered as endemic species in Europe resulting from species radiation driven by adaptation to different Fagaceae hosts [6].

With 11 *Phytophthora* species from five phylogenetic clades, the four thermo-Mediterranean riparian forest stands located at altitudes between ca. 4 and 430 m a.s.l. showed the highest *Phytophthora* diversity. In contrast, despite the higher number of 11 sampled forest stands and the wide altitudinal range between ca. 700 and 1900 m a.s.l., the meso- and supra-Mediterranean forests contained only seven *Phytophthora* species from three clades. Interestingly, only three *Phytophthora* species, *P. megasperma*, *P. multivora*, and *P. plurivora*, occurred in both categories of forest stands. Similar to the La Maddalena archipelago in Sardinia [25], also in Sicily *Q. ilex* trees hosted with nine *Phytophthora* species the highest diversity of all tree species tested. The presence of a rich community of six *Phytophthora* species in the rhizosphere of *P. orientalis* trees was surprising and warrants further investigations of their potential involvement in the decline of Sicilian plane trees, in particular, in stands with the absence of the canker and wilt pathogen *Ceratocystis fimbriata* [88]. With five *Phytophthora* species, diversity in the rhizosphere of *F. oxycarpa* trees in Sicily was similar to *Fraxinus excelsior* forests in Denmark and Poland, where five *Phytophthora* species had also been recovered [26,61].

Analogous to the forest stands, altitude also had a strong influence on the diversity and composition of the *Phytophthora* populations in the rivers. While rivers below 400 m a.s.l. contained nine *Phytophthora* species from four phylogenetic clades, only two *Phytophthora* species, *P. gonapodyides*, and *P. lacustris*, from Clade 6 and, in one river, *P.* ×*cambivora* and *P. polonica* from Clades 7 and 9, respectively, could be recovered from rivers above 600 m altitude. Eight of the 12 *Phytophthora* species recovered from rivers, *P. citrophthora*, *P. gonapodyides*, *P. lacustris*, *P. multivora*, *P. plurivora*, *P. polonica*, *P. pseudocryptogea*, and *P.* sp. kelmania, were also found in rhizosphere soil of the thermo-Mediterranean riparian forest stands. In contrast, only four of the nine *Phytophthora* species found in non-flooded meso- and supra-Mediterranean forests, *P. gonapodyides*, *P. megasperma*, *P. multivora*, and *P. plurivora*, also occur in rivers. These results indicate that the mutual exchange of *Phytophthora* inoculum between river water and forest soils is largely dependent on seasonal or episodic flooding. The results also show that several typical forest *Phytophthora* species, in particular, *P. cactorum*, *P. crassamura*, *P. quercina*, and *P. psychrophila*, cannot establish in aquatic ecosystems. Similar results were found in forests and rivers in Taiwan [21].

5. Conclusions

This study demonstrated that in ecological and environmental studies the combined use of an efficient leaf baiting technique and a reliable molecular identification method is an efficient approach for studying the diversity and distribution of *Phytophthora* species in diverse protected natural ecosystems. Eleven of the 18 known *Phytophthora* species found in this survey, including *P. crassamura*, *P. frigida*, *P. hydropathica*, *P. polonica*, *P. pseudocryptogea*, *P. quercina*, *P. thermophila*, and *P.* sp. kelmania, were detected for the first time in Sicily. The findings of *P. frigida*, *P. thermophila* and

the three new species *P. vulcanica*, *P. tyrrhenica*, and *P. citricola* 12 are first-time records for Europe. Another four species, *P. cactorum*, *P. citrophthora*, *P. megasperma*, and *P. multivora*, were previously only recorded in Sicily from nurseries or ornamental and horticultural plantings, but not from natural environments [32,67,75,89,90]. *Phytophthora cactorum*, *P. plurivora*, *P. multivora*, and *P.* ×*cambivora* are exotic, invasive wide-host-range pathogens with high aggressiveness to many native European tree species. Since their widespread occurrence in protected natural areas in Sicily poses a serious threat to the long-term stability of the infested ecosystems, management concepts are urgently required to prevent further spread of these pathogens to non-infested areas and to increase tree vigor and ecosystem stability.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/3/259/s1. Table S1: Geographic location, geomorphological features, land area covered and ecological features of the 10 Protected Natural Areas included in the *Phytophthora* survey in Sicily; Table S2: Isolate details and GenBank accession numbers of *Phytophthora* isolates obtained during the Phytophthora survey of forest stands and river systems in 10 Protected Natural Areas in Sicily; Figure S1: Geographical location of Protected Natural Areas included in the *Phytophthora* survey of forest stands and river systems in Sicily, projected using the Universal Transverse Mercator (UTM); Figure S2: Distribution of phylogenetic *Phytophthora* Clades in sampled forest stands and protected natural areas in Sicily. Brown color represents the presence of a clade. (a) Etna RP = Etna Regional Park; Nebrodi RP = Nebrodi Regional Park; Madonie RP = Madonie Regional Park; Pantalica RNR = Pantalica, Valle dell'Anapo e Torrente Cavagrande Regional Natural Reserve (RNR); Ciane RNR = Fiume Ciane e Saline di Siracusa RNR; Cavagrande RNR = Cavagrande del Cassibile RNR; Irminio SCI = ITA080002—Alto corso del Fiume Irminio Site of Community Importance (SCI), (b) proportion (%) of forest stands in which individual *Phytophthora* Clades were present, (c) proportion (%) of *Phytophthora* Clades present in individual sampled forest stands, (d) proportion (%) of protected natural areas in which individual *Phytophthora* Clades were present, (e) proportion (%) of *Phytophthora* Clades present in individual protected natural areas. Figure S3: Distribution of phylogenetic *Phytophthora* Clades in baited river systems and protected natural areas in Sicily. (a) Etna RP = Etna Regional Park; Nebrodi RP = Nebrodi Regional Park; Madonie RP = Madonie Regional Park; Pantalica RNR = Pantalica, Valle dell'Anapo e Torrente Cavagrande Regional Natural Reserve (RNR); Ciane RNR = Fiume Ciane e Saline di Siracusa RNR; Cavagrande RNR = Cavagrande del Cassibile RNR; Fiumedinisi RNR = Fiume Fiumedinisi e Monte Scuderi RNR; Agrò SCI = ITA030019—Tratto Montano del Bacino della Fiumara di Agrò—Site of Community Importance (SCI); Irminio SCI = ITA080002—Alto corso del Fiume Irminio SCI, (b) isolation frequency (%) of phylogenetic *Phytophthora* Clades from baiting rafts, (c) proportion (%) of Phytophthora Clades present in individual baited rivers, (d) proportion (%) of rivers from which phylogenetic *Phytophthora* Clades were isolated, (e) proportion (%) of natural protected areas from which phylogenetic *Phytophthora* Clades were isolated, (f) proportion (%) of *Phytophthora* Clades present in rivers of individual protected natural areas.

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