Diversity of *Phytophthora* Species Associated with *Quercus ilex* L. in Three Spanish Regions Evaluated by NGS

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Abstract: The diversity of *Phytophthora* species in declining *Fagaceae* forests in Europe is increasing in the last years. The genus *Quercus* is one of the most extended *Fagaceae* genera in Europe, and *Q. ilex* is the dominant tree in Spain. The introduction of soil-borne pathogens, such as *Phytophthora* in *Fagaceae* forests modifies the microbial community present in the rhizosphere, and has relevant environmental and economic consequences. A better understanding of the diversity of *Phytophthora* spp. associated with *Q. ilex* is proposed in this study by using Next Generation Sequencing (NGS) in six *Q. ilex* stands located in three regions in Spain. Thirty-seven *Phytophthora* phylotypes belonging to clades 1 to 12, except for clades 4, 5 and 11, are detected in this study, which represents a high diversity of *Phytophthora* species in holm oak Spanish forests. *Phytophthora chlamydospora*, *P. citrophthora*, *P. gonapodyides*, *P. lacustris*, *P. meadii*, *P. plurivora*, *P. pseudocryptogea*, *P. psychrophila* and *P. quercina* were present in the three regions. Seven phylotypes could not be associated with known *Phytophthora* species, so they were putatively named as *Phytophthora* sp. Most of the detected phylotypes corresponded to terrestrial *Phytophthora* species but aquatic species from clades 6 and 9 were also present in all regions.

Keywords: forest disease monitoring; oomycetes; natural ecosystems; holm oak decline

1. Introduction

The genus *Phytophthora* comprises nowadays more than 150 species with a broad host range, and includes well-known plant pathogens that are devastating natural ecosystems [1–3]. *Fagaceae* forests, with *Phytophthora* hosts such as *Quercus*, *Castanea*, *Lithocarpus* or *Fagus* species, are an example of declining forests affected by *Phytophthora* worldwide [4–26]. In Europe, *Castanea*, *Fagus* and *Quercus* are the most extended *Fagaceae* genera [13]. The diversity of *Phytophthora* species found in the last thirty years associated with the rhizosphere of these genera, reveals a complex syndrome in the decline of these forests [10,13,27–31]. On one hand, there is the difficulty of diagnosing which *Phytophthora* species present is the primary pathogen. On the other hand, it is also known that depending on which *Phytophthora* species is established first, the damage on the host varies [32]. Furthermore, all *Phytophthora* species present in the rhizosphere compromise forests regeneration, as they cause seedling damping off [13]. Within the genus *Phytophthora* we can find typical species of riparian ecosystems and/or water bodies that belong to clades 6 and 9. These species behave as saprotrophs or opportunistic organisms, causing in some cases tree decline, nevertheless its role is not well understood [32–35]. The remaining *Phytophthora* species included in the other clades (1 to 12, except 6 and 9) have a terrestrial life cycle, although they often end up in watercourses by runoff [13,35–37]. Either aquatic or terrestrial *Phytophthora* species are related to *Fagaceae* forests' decline [13].

Global trade increases the risk of unnoticed introductions of alien species into natural ecosystems [38,39]. The introduction of soil-borne pathogens, such as *Phytophthora* in *Fagaceae* forests modifies the microbial community present in the rhizosphere, and has relevant environmental and economic consequences [3]. Soil properties, land use, environmental conditions, the host plants and/or the microbial background determine the microbiota composition from a site [40]. Introduced pathogens have to compete with other microorganisms for available resources, which can lead to a decrease of the native microbiota. Hosts that co-evolved with soil microorganisms can adapt more easily to biotic and abiotic stresses, but a shift in the microbiota structure can trigger the host decline [3,40].

Next generation sequencing (NGS) technologies have stood out as an essential tool for environmental and ecological studies [41,42]. Pyrosequencing the Internal Transcribed Spacer (ITS1) is an efficient and accurate NGS technique for the detection and identification of *Phytophthora* spp. in environmental samples [29,35,43–45]. A better understanding of the diversity of *Phytophthora* spp. associated with a host can be provided by metagenomics, even if these *Phytophthora* spp. are not the most prevalent pathogens [3,45–47]. Nevertheless, a holistic study, combining biological and molecular identification tools, can substantially improve the diagnosis, because in many cases establishing species boundaries via molecular methods it is not an easy task [2,39,43]. In this context, it is interesting to note that samples from the current study were previously subjected to traditional isolation, baiting and real-time polymerase chain reaction (PCR) methods to detect *Phytophthora* spp. [48]. The objective of the present study was to unravel the *Phytophthora* community present in these previously studied areas using NGS technology. Thus, the diversity and abundance of *Phytophthora* spp. in six holm oak forests located in southwestern and eastern Spain were investigated using an amplicon pyrosequencing approach and the implications are further discussed.

2. Materials and Methods

2.1. Study Site and Sampling

The six study areas are holm oak forests located in three different Spanish regions: Holm oak rangelands ("dehesas") in Extremadura in southwestern Spain (province of Cáceres), four holm oak stands in the Comunidad Valenciana region in eastern Spain (two in Valencia province, one in Castellón province and one in Alicante province) and one holm oak stand in Cataluña in northeastern Spain (Barcelona province). Samplings were conducted in the autumn (fall) and winter season in different years (2012–2015), and in some areas, it was repeated for two consecutive years.

Soil samples (0.5–1 kg approx.) were collected in all the surveys, consisting in a mixture of four subsamples taken from four different points 1 m around the selected holm oak. In the surveys conducted from 2013 to 2015, along with the soil samples, roots from the rhizosphere were also taken to be analyzed. Soil and roots samples were conserved at 5 °C until DNA extraction was performed. Baiting with leaflets of *Rhododendron* sp., *Viburnum tinus* L, *Quercus ilex* L, *Quercus suber* L, *Ceratonia siliqua* L. and/or *Dianthus caryophyllus* L. petals was performed as described in a previous study [48], and the vegetal material from the baitings was conserved at -80 °C until DNA extraction.

In the Extremadura region in 2012, 60 soil samples from the declining and non-declining *Q. ilex* rhizosphere were collected from 10 "dehesas" during the autumn. In 2013, 54 soil and root samples and 216 baiting samples from 15 "dehesas" were analyzed. In the Comunidad Valenciana region, during 2014 and 2015, holm oak stands were surveyed, generating 26 soil and roots samples and 104 baiting samples from declining trees. In the Cataluña region in 2013, 15 soil and root samples and 45 baiting samples were processed from declining holm oaks.

2.2. DNA Extraction, PCR, and Preparation of the Amplicon Libraries

Each soil sample was passed through a 2 mm sieve to remove the organic matter and gravel. Once it was homogenized, 50–80 g per subsample was lyophilized overnight and pulverized using a FRITSCH Variable Speed Rotor Mill-PULVERISETTE 14 (ROSH, Oberstein, Germany). DNA from

each sample was extracted by duplicate with the Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer´s protocol. Roots and baiting vegetal material samples were firstly ground using a mortar and pestle under liquid nitrogen.

Healthy leaflets of the different vegetal species used as baitings were included as negative controls. Once homogenized, DNA extraction was performed from 60–80 mg of material per sample using the Power Plant Pro DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA).

Amplicon libraries were generated with a nested polymerase chain reaction (PCR) according to the methodology described by Català et al. [35]. The expected size of the amplicons ranged from 280 up to 450 bp. The duplicate amplicons obtained from each sample were pooled if they were positive. Negative samples were retried, firstly diluting 10 times the first round PCR product for the second round PCR. If the retried samples kept on being negative, DNA was diluted ten times, and nested PCR was performed without diluting the first round product. Positive amplicons were double purified using the Agencourt AMPureXP Bead PCR Purification protocol (Beckman Coulter Genomics, MA, USA). Samples were sequenced on the GS Junior 454 system (Roche 454 Life Science, Brandford, CT, USA) at the genotyping service facility from the Universitat de València (Burjassot, Spain).

2.3. Bioinformatics Processing and Statistical Analysis

The sequence reads were processed as described in Català et al. [35]. MOTU (Molecular Operational Taxonomic Unit) clustering of the reads was done with the 90% length coverage threshold and a 99% score coverage threshold using BLASTCLUST software [49]. The consensus sequences of the MOTUs were identified using BLAST tool in the *Phytophthora* Database [50], the MegaBLAST tool from the GenBank database [49] and a customized database. Afterwards, our query sequences from each survey and type of material (soil/vegetal) were aligned separately using MUSCLE [51]. Phylogenetic analyses consisting of maximum likelihood were performed in MEGA 6.06 [52] with the suggested suitable model, where gaps and missing data were treated as complete deletions. The robustness of the topology was evaluated by 1000 bootstrap replications [53].

A *Phytophthora* operational taxonomic unit (OTU) table was created excluding other genus reads, unaligned sequences and singletons. OTUs represented globally by less than five reads were removed [54]. The resulting quality-filtered dataset was used for the assessment of diversity and richness. The relationship in OTU composition among samples were investigated by calculating Bray Curtis metrics, and visualized by means of PCoA plots. Samples without *Phytophthora* reads were excluded for the diversity analyses. Biodiversity indices and principle statistics analyses on taxonomic profiles were analyzed in the tool MicrobiomeAnalyst [55].

3. Results

3.1. Sequencing Results

In the Extremadura region, 66,732 total ITS1 sequences were generated from the pyrosequenced soil samples collected in 2012. These had an average length of 308 bp, and only 61,576 high quality sequences were considered for the analysis after trimming, and excluding singletons; 48.7% of the sequences were identified from declining trees, and 51.3% from non-declining trees.

From samples collected in 2013 in the Extremadura region, 377,799 ITS1 sequences reads were obtained with an average length of 302 bp. After trimming and excluding singletons, 317,961 high quality sequences were used for the analysis; 103,333 from the root samples (45.05% from declining trees and 54.95% from non-declining trees), 56,112 sequences from the soil samples (45.3% from declining trees and 54.7% from non-declining trees) and 158,516 sequences from the baiting samples (51.68% from declining trees and 48.32% from non-declining trees). No *Phytophhtora* phylotypes are detected from the negative baiting controls.

In the Cataluña region, 63,105 total ITS1 sequences were generated from the pyrosequenced samples. These had an average length of 283 bp, and only 55,230 high quality sequences were

considered for the analysis after trimming and excluding singletons (28,381 from roots, 9887 from soils and 16,962 from baitings).

In the Comunidad Valenciana region, 177,398 ITS1 raw sequences with an average length of 309 bp were obtained. After trimming and excluding singletons, 78,962 high quality sequences were considered for the analysis (3,977 from sampled roots, 18,861 from soils and 56,124 from baitings).

3.2. Identification of Phytophthora Phylotypes

3.2.1. Extremadura Region

A total of 33 different *Phytophthora* phylotypes are identified in the Extremadura region during the surveys conducted in 2012 and 2013 (Table 1). Detected phylotypes belong to all clades except for clades 4, 5 and 11, the clade 6 having the highest number of *Phytophthora* phylotypes detected (Table 1). In 2012, 20 *Phytophthora* phylotypes were obtained, and 15 of them identified to the species level: *Phytophthora bilorbang* Aghighi, G.E. Hardy, J.K. Scott & T.I. Burgess, *Phytophthora botryosa* Chee, *Phytophthora cambivora* (Petri) Buisman, *Phytophthora chlamydospora* Brasier and Hansen, *Phytophthora cinnamomi* Rands., *Phytophthora cryptogea* Pethybr. and Laff., *Phytophthora gemini* Man in 't Veld, Rosendahl, Brouwer and de Cock, *Phytophthora gonapodyides* (H.E. Petersen) Buisman, *Phytophthora hydropathica* Hong and Gallegly, *Phytophthora lacustris* Brasier, Cacciola, Nechwatal, Jung and Bakonyi, *Phytophthora lagoariana*, *Phytophthora multivora* Scott and Jung, *Phytophthora plurivora* Jung and Burgess, *Phytophthora psychrophila* Jung and Hansen, *Phytophthora quercina* Jung and *Phytophthora riparia* Reeser, Sutton and Hansen. One phylotype belong to complex *Phytophthora uliginosa-europaea* as the ITS1 region used in the assay could not resolve its identity. Three phylotypes do not correspond to any *Phytophthora* sequence included in databases; therefore, these putative new species are named *Phytophthora* sp.1, *Phytophthora* sp.2 and *Phytophthora* sp.3.

In 2013, 25 *Phytophthora* phylotypes were recovered in Extremadura and 22 of them were identified to the species level: *Phytophthora* taxon *ballota*, *P. bilorbang*, *P. chlamydospora*, *P. cinnamomi*, *Phytophthora citrophthora* (R.E. Sm. and E.H. Sm.) Leonian, *Phytophthora clandestina* Taylor, Pascoe and Greenhalgh, *P. cryptogea*, *Phytophthora gallica* Jung and Nechwatal, *P. gonapodyides*, *P. hydropathica*, *Phytophthora insolita* Ann and Ko, *Phytophthora* sp. *kelmania*, *P. lacustris*, *Phytophthora lactucae* Bertier, Brouwer and de Cock, *P. lagoariana*, *Phytophthora meadii* McRae, *Phytophthora megasperma* Drechsler, *Phytophthora* sp. *palustris*, *P. plurivora*, *Phytophthora pseudocryptogea* Safaiefarahani, Mostowfizadeh, G.E. Hardy, and T.I. Burgess, *P. psychrophila*, *P. quercina* and *Phytophthora rosacearum* Hansen and Wilcox. The ITS1 region used in the assay could not resolve the identity of one *Phytophthora* phylotype complex: *Phytophthora uliginosa-europaea*. One *Phytophthora* phylotype does not match with any *Phytophthora* sequence included in the databases, therefore this putative new species is named as *Phytophthora* sp.4.

Table 1. *Phytophthora* phylotypes detected by next generation sequencing (NGS), based on the Internal Transcribed Spacer (ITS1) region.

Phylotypes	Clade	Spanish Regions					
		Extremadura	Cataluña	Comunidad Valenciana			
				Font Roja	Hunde	Pina	Alcublas
BAL	1	√		√	√		
BIL	6	√					
BOT	2	√					
CAM	7	√					
CHL	6	√	√			√	√
CIN	7	√	√				
CIP	2	√	√			√	
CLA	1	√					
CRY	8	√					
GAL	10	√					
GEM	6	√					
GON	6	√	√				√
HYD	9	√	√				
INS	9	√	√				
KEL	8	√				√	
LAC	6	√	√			√	√
LCT		√					
LAG	9	√	√				
MEA	2	√	√			√	
MEG	6	√					√
MUL	2	√					
PAS	9	√					
PLU	2	√	√			√	√
PSC	8	√	√		√	√	√
PSY	3	√	√	√	√		√
QUE	12	√	√	√	√	√	
RIP	6	√					
ROS	6	√					
TEN	1						√
ULIG-EUR	7	√		√			
SP.1	6	√					
SP.2	7	√					
SP.3	8	√					
SP.4	1	√					
SP.5	7		√				
SP.6	3			√			
SP.7	1						√

BAL, *P.* taxon *ballota*; BIL, *P. bilorbang*; BOT, *P. botryosa*; CAM, *P. cambivora*; CHL, *P. chlamydospora*; CIN, *P. cinnamomi*; CIP, *P. citrophthora*; CLA, *P. clandestina*; CRY, *P. cryptogea*; GAL, *P. gallica*; GEM, *P. gemini*; GON, *P. gonapodyides*; HYD, *P. hydropathica*; INS, *P. insolita*; KEL, *P. sp. kelmania*; LAC, *P. lacustris*; LCT, *P. lactucae*; LAG, *P. lagoariana*; MEA, *P. meadii*; MEG, *P. megasperma*; MUL, *P. multivora*; PAS, *P.* sp. *palustris*; PLU, *P. plurivora*; PSC, *P. pseudocryptogea*; PSY, *P. psychrophila*; QUE, *P. quercina*; RIP, *P. riparia*; ROS, *P. rosacearum*; TEN, *P. tentaculata*; ULIG-EUR, *P. uliginosa-P. europaea*; SP.1-SP.7, new phylotypes found not identified to the species level.

3.2.2. Cataluña Region

Fourteen *Phytophthora* phylotypes are identified in the Cataluña region during the survey conducted in 2013, belonging to clades 2, 3, 6, 7, 8, 9 and 12 (Table 1). Thirteen phylotypes are identified to the species level: *P. chlamydospora*, *P. cinnamomi*, *P. citrophthora*, *P. gonapodyides*, *P. hydropathica*, *P. insolita*, *P. lacustris*, *P. lagoariana*, *P. meadii*, *P. plurivora P. pseudocryptogea*, *P. psychrophila* and *P. quercina*. One *Phytophthora* phylotype does not match with any *Phytophthora* sequence included in the databases, therefore this putative new species is named *Phytophthora* sp.5.

3.2.3. Comunidad Valenciana Region

The *Phytophthora* phylotypes identified in Comunidad Valenciana during the surveys conducted in the period 2014-2015 is shown in Table 1. Sixteen *Phytophthora* phylotypes are identified in Comunidad Valenciana belonging to clades 1, 2, 3, 6, 7, 8 and 12 (Table 1). Thirteen phylotypes are identified to the species level: *P.* taxon *ballota, P. chlamydospora, P. citrophthora, P. gonapodyides, P.* sp. *kelmania, P. lacustris, P. meadii, P. megasperma, P. plurivora P. pseudocryptogea, P. psychrophila, P. quercina* and *Phytophthora tentaculata* Kröber and Marwitz. The ITS1 region used in the assay could not resolve the identity of one *Phytophthora* phylotype complex: *P. uliginosa-europaea*. Two *Phytophthora* phylotypes do not match with any *Phytophthora* sequences included in the databases and thus, these putative new species are named as *Phytophthora* sp.6. and *Phytophthora* sp.7.

3.2.4. Phytophthora Diversity

The relative abundance of *Phytophthora* species detected from 2013 to 2015 in the three studied regions is shown in Figure 1A. The Extremadura region presents the greatest diversity of *Phytophthora* species. According to the type of material used for *Phytophthora* isolation, baiting material has the highest relative abundance of *Phytophthora* species, followed by roots and soils (Figure 1B).

Regarding symptomatology, non-declining holm oak samples in the Extremadura present a higher diversity of *Phytophthora* species either in the 2013 survey or analyzing 2012 and 2013 together, than samples from declining trees (Figure 2A,C), but this diversity was slightly higher in declining samples in the 2012 survey (Figure 2B).

The ANOVA shows that the alpha diversity was significantly higher in the Extremadura region, followed by Comunidad Valenciana and Cataluña (Figure 3). Both the Chao 1 estimator (p value < 0.05) and the Shannon index (p value < 0.01) were significant according to the factor region (Table 2). Regarding the factor material, the Shannon index was significant (p value = 0.033), showing greater diversity of *Phytophthora* species when using baiting material, while the Chao 1 estimator was non-significant (p value = 0.091) (Table 2).

Table 2. Analysis of variance (ANOVA) table for the alpha diversity of *Phytophthora* species detected in the study.

	α **Diversity**		
	Region	**Material**	**Region $\times$ Material**
Chao 1 estimator	$F_{2,245} = 10.8$ $p \leq 0.01$	$F_{2,196} = 1.9$ $p = 0.091$	$F_{4,188} = 5.5$ $p \leq 0.01$
Shannon index	$F_{2,245} = 7.1$ $p \leq 0.01$	$F_{2,196} = 2.3$ $p = 0.033$	$F_{4,188} = 4.7$ $p \leq 0.01$

In the Extremadura region where the factor symptomatology was studied, in 2012 alpha diversity measured by the Chao1 estimator results was significant for the factor symptomatology (p value = 0.0218), but the Shannon Index was non-significant (p value = 0.1303). Nevertheless, in 2013 the ANOVA shows that both estimators were significant for the factor material and non-significant for the factor symptomatology (Table 3).
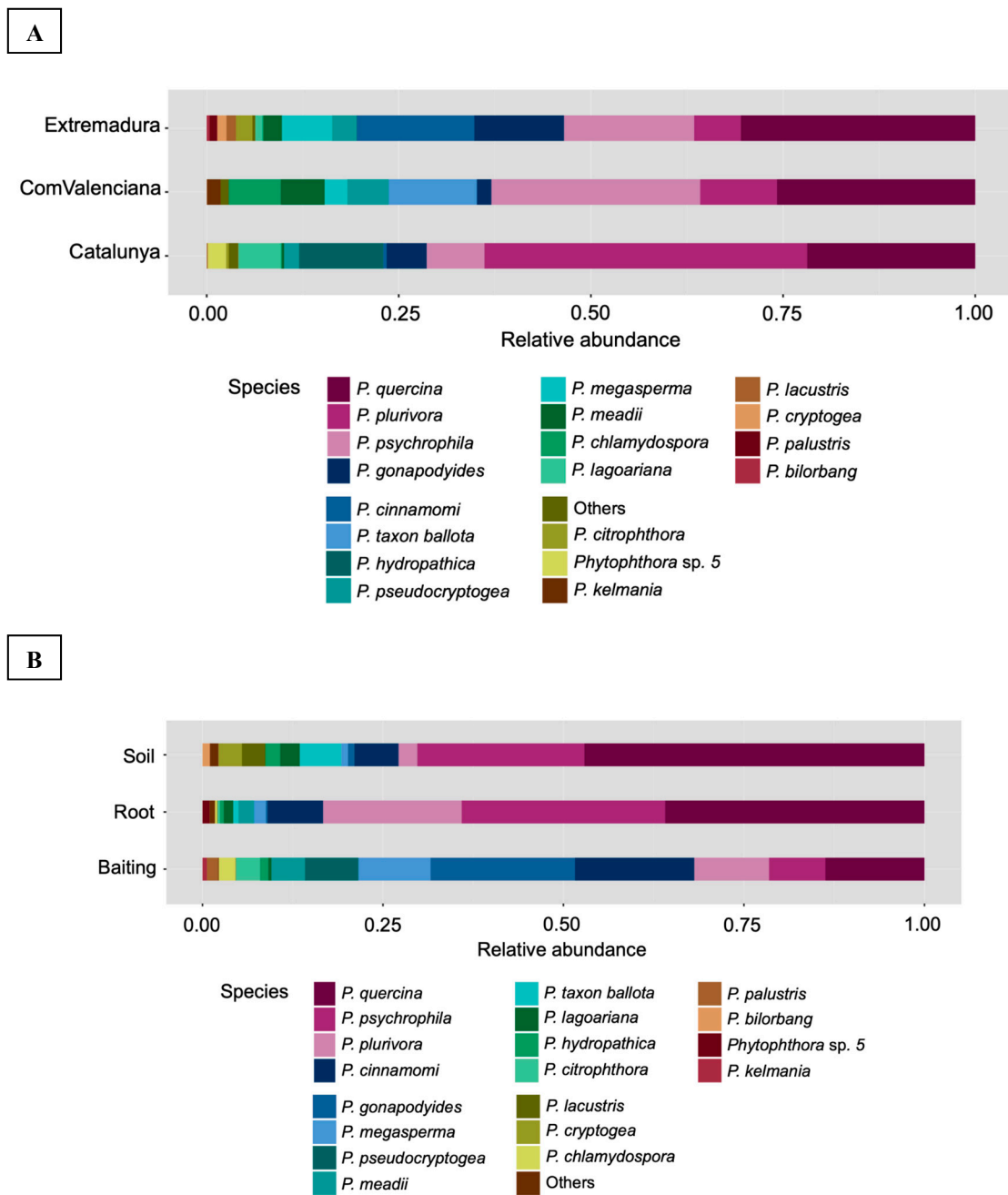
Figure 1. Relative abundance of *Phytophthora* species detected in the three studied regions from 2013 to 2015 showing more than 1% relative abundance of all reads. Species representing less than 1% of the total reads are grouped in "others". (**A**): According to the factor region; Extremadura, Comunidad Valenciana (ComValenciana) and Cataluña (Catalunya). (**B**): According to the factor type of material; soils, roots and baitings. Results of the survey performed in Extremadura in 2012 are excluded in this analysis, since only soil samples were included.
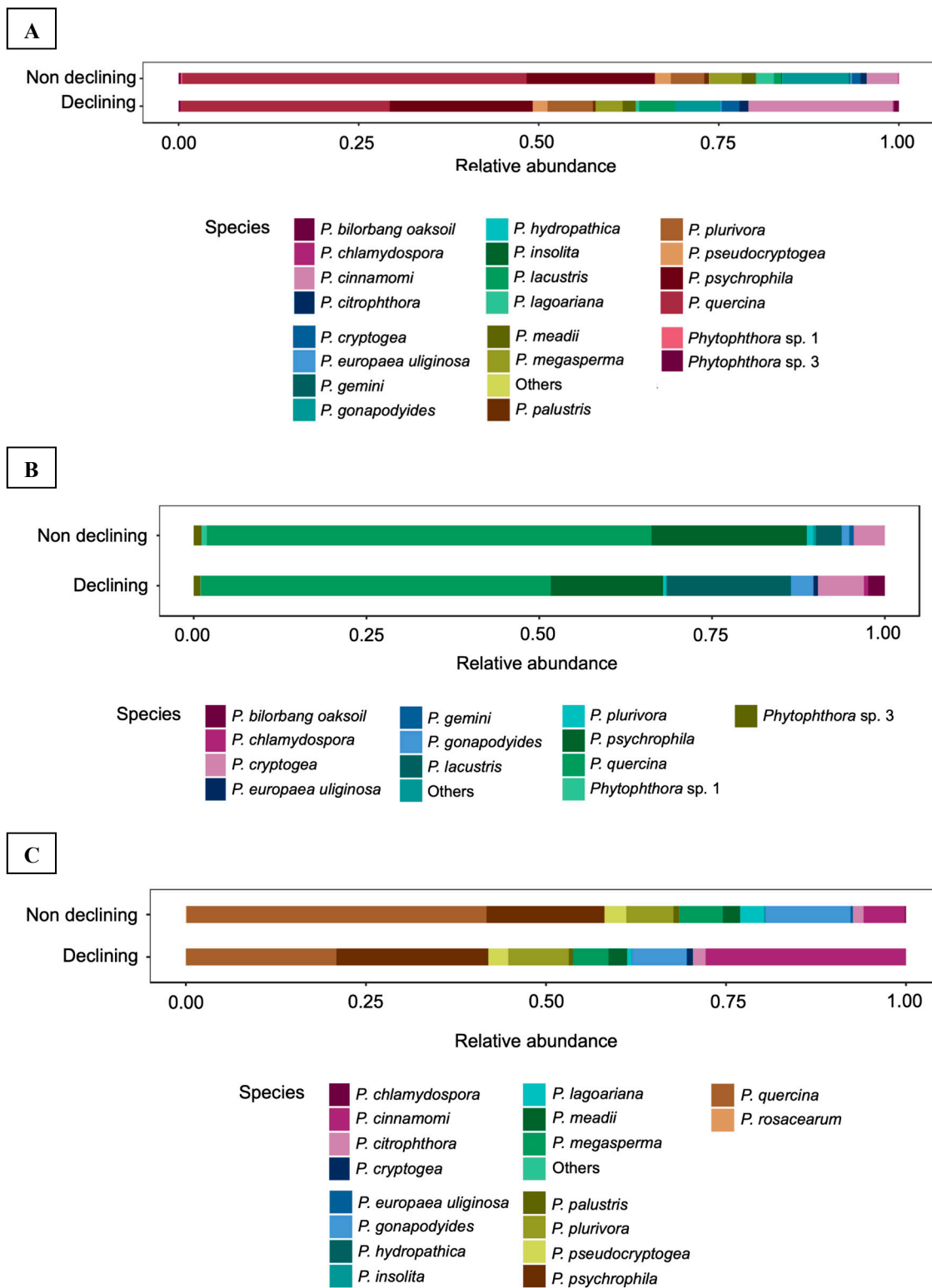
Figure 2. Relative abundance of *Phytophthora* species showing more than 1% relative abundance of all reads in Extremadura region according to the factor symptomatology. Species representing less than 1% of the total reads are grouped in others. (**A**): During the surveys conducted in 2012 and 2013. (**B**): Survey conducted in 2012. (**C**): Survey conducted in 2013.
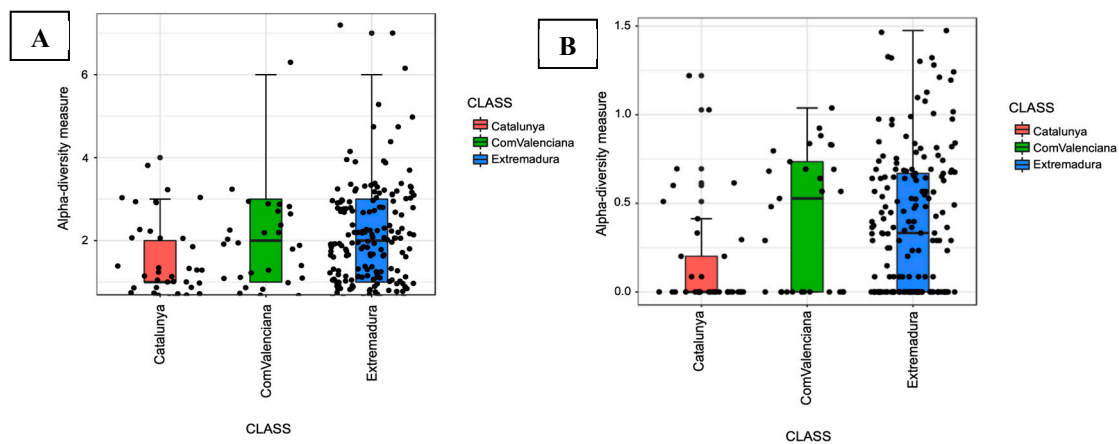
Figure 3. Boxplot showing alpha diversity measures of the *Phytophthora* diversity according to the factor region: Extremadura, Comunidad Valenciana (ComValenciana) and Cataluña (Catalonia). (**A**): Chao 1 estimator (*p* value < 0.05). (**B**): Shannon index (*p* value < 0.01).

Table 3. Analysis of variance (ANOVA) tables for the alpha diversity of *Phytophthora* species detected in Extremadura dehesas during the surveys conducted in 2012 and 2013.

| Survey | α Diversity | | | | | |
| | Chao 1 Estimator | | | Shannon Index | | |
	Material	Symptoms	Material × Symptoms	Material	Symptoms	Material × Symptoms
2012	ndt	$F_{1,47}= 2.39$ $p = 0.0218$	ndt	ndt	$F_{1,47}= 1.54$ $p = 0.1303$	ndt
2013	$F_{2,126} = 6.03$ $p = 0.0029$	$F_{1,127} = 1.23$ $p = 0.2199$	$F_{2,123} = 2.7$ $p = 0.023$	$F_{2,126} = 4.95$ $p = 0.0081$	$F_{1,127} = 0.83$ $p = 0.4085$	$F_{2,123} = 2.4$ $p = 0.041$

ndt = not determined.

The principal coordinates analysis (PCoAs) of Bray Curtis show that the *Phytophthora* population in Extremadura had more variation in the number of species present, although there were similarities with the populations of the other two regions (Figure 4A). There are no significant differences in the diversity of *Phytophthora* species detected in the type of material used or the symptomatology of the sampled trees (Figure 4B,C).

Nine *Phytophthora* phylotypes were detected in the three regions (24.3%): *P. citrophthora*, *P. meadii*, *P. plurivora*, *P. psychrophila*, *P. quercina*, *P. chlamydospora*, *P. gonapodyides*, *P. lacustris* and *P. pseudocryptogea* (Figure 5A). Regarding the type of material, 11 *Phytophthora* phylotypes were common to root, soil and baiting material (28.9%): *P. plurivora*, *P. psychrophila*, *P. quercina*, *P. chlamydospora*, *P. gonapodyides*, *P. lacustris*, *P. megasperma*, *P. cinnamomi*, *P.* uliginosa - europaea, *P. insolita* and *P. lagoariana* (Figure 5B). Finally, 20 *Phytophthora* phylotypes were present in declining and non-declining holm oak trees, which represent 54.05% of the detected *Phytophthora* phylotypes (Figure 5C).
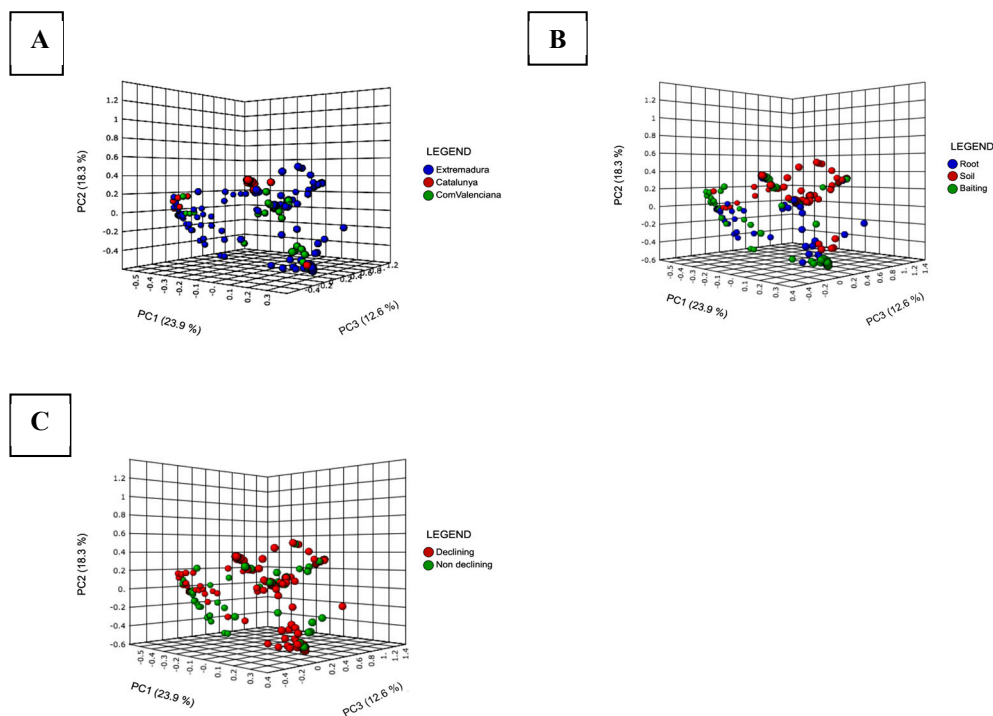
Figure 4. Principal Coordinate Analysis (PCoA) based on Bray Curtis dissimilarity metrics, showing the distance in the *Phytophthora* spp. composition according to the different factors studied. (**A**): Regions surveyed; Extremadura, Comunidad Valenciana (ComValenciana) and Cataluña (Catalunya). (**B**): Type of material used for the detection; roots, soils and baitings. (**C**): Symptomatology of the holm oaks sampled: declining and non-declining.
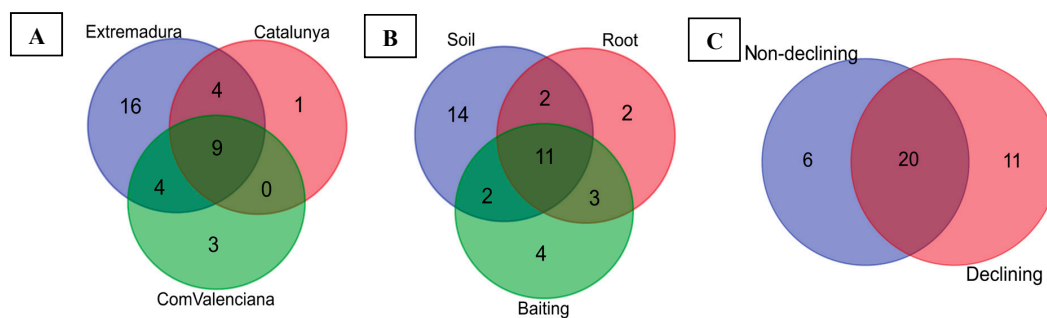
Figure 5. Venn diagram showing the overlap of operational taxonomic units (OTUs) identified in the *Phytophthora* population present in the holm oaks surveyed in the study according to the different factors studied. (**A**): Regions surveyed; Extremadura, Comunidad Valenciana (ComValenciana) and Cataluña (Catalunya), (**B**): Type of material used for the detection; roots, soils and baitings. (**C**): Symptomatology of the holm oaks sampled: declining and non-declining.

4. Discussion

Thirty-seven *Phytophthora* phylotypes belonging to clades 1 to 12, except for clades 4, 5 and 11, were detected in this study, which represents a high diversity of *Phytophthora* species in holm oak Spanish forests compared to previous Spanish NGS studies [44,56]. *Phytophthora chlamydospora*, *P. citrophthora*, *P. gonapodyides*, *P. lacustris*, *P. meadii*, *P. plurivora*, *P. pseudocryptogea*, *P. psychrophila* and *P. quercina* were present in the three regions. Seven of these phylotypes cannot be associated with known *Phytophthora* species, so they are putatively named as *Phytophthora* sp. Most of the detected phylotypes correspond to terrestrial *Phytophthora* species, but aquatic species from clades 6 and 9 were also present in all regions. In general, the most abundant phylotypes in the study are *P. quercina*,

followed by *P. psychrophila*, *P. cinnamomi* and *P. plurivora*; all of them terrestrial species. Our results concur with Català et al. [44], which reported *P. quercina* and *P. psychrophila* as the most relevant species associated with *Q. ilex* in eastern Spain. Nevertheless, they differ from Ruiz Gómez et al. [56] describing *P. plurivora*, *P. quercina*, *P. cinnamomi* and *P. cactorum* as the most frequent detected species in Andalucía holm oak rangelands (dehesas) located in southern Spain.

In the Extremadura region, all *Phytophthora* clades are present with the exception of clades 4, 5 and 11. Terrestrial species dominate the *Phytophthora* community as *P. quercina* 30%, *P. psychrophila* 17% and *P. cinnamomi* 15%, are the most abundant species. Nevertheless, also aquatic species are identified, such as *P. gonapodyides* 12% and *P. megasperma* 7%, which follow in abundance. Hence, a mixture of *Q. ilex* pathogenic terrestrial and aquatic species is identified in the Extremadura region. In the Cataluña region, the *Phytophthora* community associated with the studied *Q. ilex* stand was dominated again by terrestrial species (*P. plurivora* 42%, *P. quercina* 22% and *P. psychrophila* 8%), followed by aquatic species (*P. hydropathica* 11%, *P. lagoariana* 6% and *P. gonapodyides* 5%). Clades 1, 4, 5, 8, 10 and 11 were not detected in the studied holm oaks. In the Comunidad Valenciana region, the *Phytophthora* community is made up primarily by terrestrial *Phytophthora* species from clades 1, 2, 3, 7, 8 and 12 (*P. psychrophila* 27%, *P. quercina* 26%, *P.* taxon *ballota* 11%, *P. plurivora* 10%); although there are also present in lower abundance aquatic species from clade 6 such as *P. chlamydospora* 7%, *P. gonapodyides* 2% or *P. megasperma* 3%.

The weather conditions of the Extremadura region were more favorable for *Phytophthora* development in 2013 than in 2012, since in 2012 Extremadura received less precipitation than in 2013, as reported in 2018 by Mora-Sala et al. [48]. Redondo et al. [57] state that there is a decrease in the diversity of terrestrial *Phytophthora* communities when temperature and precipitation decreases, precipitation being the main driving factor, except for clades 2 and 8, in which temperature was more conditioning. According to Redondo et al. [57], aquatic *Phytophthora* species are inversely conditioned by temperature and precipitation. The *Phytophthora* diversity found in Extremadura in 2013 was higher than in 2012, which fits with the parameters stated in the study of Redondo et al. [57]. Moreover, more types of sampling materials (roots and baitings) were used for DNA extraction in 2013 than in 2012, which might potentially increase the phylotype diversity. Redondo et al. [57] conclude that the land use is only significant for aquatic species, having more diversity in urban or agricultural sites than in forests. In our study, we found more species belonging to the clade 6 in Extremadura oak rangelands, such as *P. gonapodyides* or *P. megasperma*, which are man-made forests, although they are also identified in lower abundance in Cataluña and Comunidad Valenciana. Clade 9 species (*P. hydropathica* and *P. lagoariana*) are also detected in Extremadura and in Cataluña.

Phytophthora cinnamomi is an important and devastating *Quercus* pathogen in Spain [27,58–61], although it is not the most abundant species detected in the *Quercus* forests studied. The pathogen is significantly more abundant in declining trees than in non-declining trees, as previously reported by Mora-Sala et al. using qPCR and traditional isolation methods [48], and that also corroborates the metabarcoding study of Ruiz Gómez et al. [56]. *Phytophthora cinnamomi* is not detected in Comunidad Valenciana.

This is in agreement with previously published reports, and may be due to the unsuitable conditions for the pathogen development in the area consisting on primarily calcareous soils with a high pH [26,44,48,62].

Phytophthora quercina is a specific oak pathogen, present in the three studied regions, that probably has co-evolved with *Q. ilex* in Spain, rotting the trees slowly and progressively. As *P. quercina* was not described until 1999 [8], it could not be associated with the studies of the decline of Spanish holm oak conducted in the past. In addition, *P. quercina* is a difficult pathogen to isolate, due to its slow growth, thus its detection was not always possible in previous studies [8,15,26,30]. Moreover, as generally *P. cinnamomi* was present, and *P. quercina* is not a fast growing pathogen, the decline was associated with *P. cinnamomi*. Although recent studies report the presence of *P. quercina*, it is thought of as not being as frequent as *P. cinnamomi*. Mora-Sala et al. demonstrate that *P. quercina* is more frequent than

P. cinnamomi, not only in Comunidad Valenciana, but also in the Extremadura and Cataluña regions by qPCR [48]. Ruiz Gómez et al. [56] report *P. quercina* as the fourth-most abundant oomycete in Andalucía holm oak rangelands, while *P. cinnamomi* stands in the ninth position. The present study based on amplicon pyrosequencing supports our previous results using qPCR and verifies that this pathogen is highly abundant in the studied *Quercus* Spanish regions.

Phytophthora psychrophila is detected in high abundance in the three regions sampled, especially in Comunidad Valenciana, either in declining or in non-declining trees. This species was previously related to *Quercus* spp. dieback in Spain where it is apparently well distributed and it is only able to be isolated during the winter and spring/autumn periods [10,26,56]. As Pérez-Sierra et al. report in 2013, it is perfectly adapted to xeric Mediterranean conditions due to the thick wall of its resting structures that enables it to overcome unsuitable environmental conditions. Pathogenicity tests with *Q. ilex* seedlings demonstrate that *P. psychrophila* is considered an aggressive pathogen, causing dieback of the root system, mainly the fine roots, necrotic lesions and open cankers [26,48].

Phytophthora plurivora is significantly detected in roots and it is even present in all regions, resulting more abundantly in the Cataluña region, where it is the most frequent detected species. *Phytophthora plurivora* is a widespread species in Europe, which had been already detected in *Q. ilex* in Spain [44,56], and its pathogenicity to *Q. ilex* is demonstrated in a previous study [63] causing the absence of fine roots, necrotic lesions, open cankers, dieback of the whole root system and collar rot. Ruiz Gómez et al. [56] report *P. plurivora* as the most abundant oomycete detected in the Spanish oak rangelands surveyed in their study. *Phytophthora plurivora* in our study seems to be more associated with declining *Q. ilex* trees, as Ruiz Gómez et al. [56] report. The homothallic behavior of *Phytophthora* species, as *P. plurivora* or *P. psychrophila*, facilitates its reproduction and establishment in new areas, boosting the risk of forest decline [10,26,29,36,57,64].

From the aquatic species detected, *P. gonapodyides* was previously detected affecting *Q. ilex* in Spain [26,33,48]. *Phytophthora megasperma* is reported to reduce the root system [26,63] although this species is considered an opportunistic oak pathogen present in oak forests [9,15,16]. The role of the remaining aquatic *Phytophthora* species detected in the *Q. ilex* decline is still unknown.

The sequencing results support the presence of *P.* taxon *ballota* in two forests of the Comunidad Valenciana and in Extremadura regions. This uncultured phylotype was previously detected in oak forests in Comunidad Valenciana [44], but it is the first time that it has been detected outside the Comunidad Valenciana. The designation of new phylotypes from environmental DNA remains a committed issue, but it is reported in other studies [65–67]. Both in previous studies and the present study, no *Phytophthora* culture was isolated that coincided with this proposed phylotype using traditional methods. Further surveys targeting this organism should be performed to try the isolation.

This study detected the presence of *P. pseudocryptogea* in all three regions, and it has also been isolated from other *Fagaceae* such as *Castanea sativa* in the North of Spain (Mora-Sala and Català unpublished), suggesting that it is well established in Spain and probably in the past many isolates identified as *P. cryptogea*, were actually *P. pseudocryptogea*. In 2018, *P. pseudocryptogea* was firstly reported in Extremadura region from *Q. ilex* rhizosphere [48] and it has just been reported in Sicily affecting *Q. ilex* [36], so it seems to be well established in the Mediterranean basin.

The pathogenicity on *Q. ilex* has not been tested, but its pathogenicity on other *Fagaceae* (Mora-Sala and Català, unpublished), suggests that this species could contribute to the oak decline.

Phytophthora community diversity recovered from *Fagaceae* forests in Europe has increased in the last years [8–10,13,16,20,21,23,24,26,29,30,36,44,64,68]. The implementation of NGS technologies to forests surveys helps to improve the knowledge about the *Phytophthora* spp. diversity associated with *Fagaceae* forests and to identify possible new introduced species [3,29,44,45,47]. *Phytophthora* spp. can adapt to a wide variety of environmental conditions [38]. In Spain, the oak decline due to *Phytophthora* spp. is related to the effect of the water stress; seasonal droughts followed by floods enhance the root damage induced by *Phytophthora* species. The versatility of these species to cope with the changing scenarios and the increase of extreme weather conditions that are occurring nowadays,

focuses the attention on this destructive genus. The composition of *Phytophthora* species in these ecosystems is changing because of their adaptation to new environmental conditions and new species introduction. New technologies help us improve knowledge about species diversity in these new scenarios. In the present study, NGS reveals a higher *Phytophthora* species diversity than previously detected by traditional isolation, baiting and qPCR. However, the best approach should combine all available methodologies for a correct *Phytophthora* diagnosis, facilitating a quick answer facing the potential introduction of new and/or quarantine organisms.

5. Conclusions

The use of amplicon pyrosequencing reveals a high diversity of *Phytophthora* species associated with *Q. ilex*. Baiting material has the highest relative abundance of *Phytophthora* species. The highest alpha diversity is obtained in the region of Extremadura, the diversity of Comunidad Valenciana and Cataluña being lower following this order. In general terms, the *Phytophthora* diversity is highest in non-declining *Q. ilex* than in declining trees. The implementation of molecular tools in *Phytophthora* forests monitoring, complement and help to overcome the limitations of traditional methods, being useful to improve the knowledge about the real composition of the species present in these ecosystems.

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