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Soil properties have more significant effects on the community composition of protists than the rhizosphere effect of rice plants in alkaline paddy field soils

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ABSTRACT

Protists are among the most diverse and major microbial groups in the soil ecosystem and play versatile functional roles for soil fertility and agricultural productivity. However, protist community composition is poorly understood in paddy field soil, especially in alkaline paddy fields. Here we aimed to characterise protist communities of alkaline paddy field soils with a particular focus on the effects of physicochemical properties of the soils and the rhizosphere effect of rice. We selected several alkaline paddy fields across three regions that differed in their soil physicochemical properties along the Kizilirmak River, Turkey, as a model ecosystem. The soils were incubated under submerged conditions with and without rice plants (*Oryza sativa* L.) to understand the rhizosphere effect on protist community composition. The protist communities were analysed with a high throughput sequencing method. The results showed that Amoebozoa (29.5%) were the most abundant taxonomic group of protists in the paddy fields, followed by Stramenopiles (23.7%), Rhizaria (19.5%), and Alveolata (12.6%). Among the functional groups, consumers (decomposers and predators) were the most dominant protist group (67.7%), followed by autotrophs (21.0%) and pathogens (microbial/animal parasites and plant pathogens) (9.2%). The soil properties have more significant effects driving the community composition of protists than the rhizosphere effect of rice in the paddy field soils. Among the soil properties, pH, exchangeable Na and Ca, EC, and lime were significantly correlated with the shift in the protist community composition. The rhizosphere effect of rice mainly affected phagotrophs and plant pathogens, especially *Pythium* sp. A significant negative correlation was observed in the relative abundances between phagotrophic protists and plant pathogens, which indicates that the plant pathogens could be top-down controlled by the phagotrophs.

1. Introduction

Protists, the vast majority of the eukaryotes, are among the most diverse and dominant microbial groups in the soil ecosystem (Geisen et al., 2018). Their taxonomic diversity results in versatile functionalities. Phagotrophic protists (microbial predators) regulate microbial populations and shape microbial communities (Gao et al., 2019). The predatory activities of phagotrophic protists alter bacterial functionalities, accelerate nutrient turnover, and increase plant nutrient uptake (Clarholm, 1985; Kuikman and Van Veen, 1989; Bonkowski, 2004). Several protists play essential roles in nutrient cycling by organic matter degradation and carbon fixation (Jassey et al., 2015; Kramer et al., 2016). Some protists are plant pathogens having enormous negative impacts on plant production. Animal and microbial parasites negatively affect their hosts' health (Latijnhouwers et al., 2003; Mahé et al., 2017). The taxonomic and functional diversity of protists provides valuable information to understand the soil ecosystem dynamics. Soil protists are highly sensitive to environmental factors and respond differently to the biotic and abiotic factors from bacteria and fungi (Geisen et al., 2018). Among the environmental factors, nitrogen fertilizer-induced changes on protist communities, especially on phagotrophs, were stronger than that on bacterial and fungal communities (Zhao et al., 2019, 2020). This

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may be partly explained by increased ammonia levels through nitrogen fertilisers, which can suppress protist growth through cell disruption (Puigagut et al., 2005; Angus et al., 2014). Along with the fertilisers, changes in the soil pH, soil moisture, and organic matter content due to agricultural land usage affect protist diversity (Santos et al., 2020). Changes in soil nutrients and porosity by biochar amendments differently affected phagotrophic and autotrophic protists (Asiloglu et al., 2021b). Scherber et al. (2010) showed that bottom-up effects of plant diversity affect higher trophic levels (phagotrophs) more strongly than the lower trophic levels (bacteria). Taken together, previous studies showed that protists are more susceptible to changes induced by environmental factors, especially soil water availability, climate (temperature and precipitation), soil nutrients, and the rhizosphere effects of plants, than their counterparts (bacteria and fungi) (Geisen et al., 2018). However, majority of the knowledge on the interaction between protists and environmental factors comes from upland fields, and less is known about the driving factors on the taxonomic and functional diversity of protists in submerged paddy fields.

Paddy field soil has distinct biogeochemical cycles from upland soils (Kirk, 2004) and provides a unique environment consisting of a different protist community than the upland fields (Asiloglu et al., 2015). Previous studies based on old-fashion molecular methods (DGGE, T-RFLP) showed that soil water and oxygen availability (Murase et al., 2014), chemical and organic fertilisers (Murase et al., 2015), and the rhizosphere effect of rice (Asiloglu et al., 2015; Asiloglu and Murase, 2016, 2017) are the main driving force controlling protist communities. In paddy fields, phagotrophic protists feed on bacteria and alter their communities (Murase et al., 2006; Asiloglu et al., 2020, 2021c). Indeed, we recently showed that the bacterial communities were top-down regulated by phagotrophic protists rather than the bottom-up regulation by fertilisers (Asiloglu et al., 2021a). The predatory activities of phagotrophic protists in paddy field soil affect the methane cycle (Murase and Frenzel, 2007, 2008), bacterial and fungal activities, especially on nitrogen-cycling (Murase et al., 2006; Herdler et al., 2008; Asiloglu et al., 2021c), and subsequently enhance the rice plant growth (Kreuzer et al., 2006; Herdler et al., 2008; Asiloglu et al., 2020, 2021c). Paddy field protists play essential roles in the carbon cycle as primary producers and decomposers (Kirk, 2004; Murase et al., 2012). In addition, protists also include some important pathogens, especially *Pythium* spp., are a serious threat to rice productivity (Furuya et al., 2003). The high-throughput sequencing method demonstrated the great diversity of soil protists and revealed the importance of the protist groups, which was previously unrecognised in the upland fields (Bates et al., 2013). However, the high-throughput sequencing method has been only recently introduced to reveal the driving factors of protist communities in paddy field soil (Asiloglu et al., 2021b).

The majority of rice plants are cultivated on acidic soils worldwide. However, depending on the rainfall distribution and water management, alkalinity may become a problem in paddy fields, especially in semiarid regions, which is often associated with soil salinity (Dobermann and Fairhurst, 2000). To our knowledge, very little is known about soil protists in alkaline paddy soils. Protists are essential players of the soil food-web and contribute to soil fertility (Geisen et al., 2018; Gao et al., 2019), which could be linked to enhanced agricultural productivity (Xiong et al., 2020; Guo et al., 2021). Therefore, understanding protist communities could provide important information on the performance of alkaline paddy field soils. Here, we aim to characterise the protist communities in alkaline paddy fields using the high-throughput sequencing method (Illumina MiSeq). Both soil properties and the rhizosphere effect of rice are major controlling factors of protist communities in acidic paddy fields (Asiloglu et al., 2015; Murase et al., 2015; Asiloglu and Murase, 2016). However, to the best of our knowledge, there are no reports describing how protist communities are shaped in alkaline paddy field soil. Here we focused on the effects of soil physicochemical properties and the rhizosphere effect of rice (*Oryza sativa* L. Osmancik 97). Previous studies in acidic paddy fields showed that protists communities are determined by soil physicochemical properties (Murase et al., 2015) and the rhizosphere effect of rice plants (Asiloglu et al., 2015; Asiloglu and Murase, 2016). Here, we hypothesised that both soil properties and the rhizosphere effect of rice plants would shape the protist communities in alkaline paddy fields as well.

Alkaline paddy fields along the Kizilirmak River in Turkey were selected as a model ecosystem. The Kizilirmak River is the longest river in Turkey and the primary water source for domestic rice production in eight cities (Çakmak et al., 2007), which covers over 25% of the total rice production of Turkey (Tasligil and Sahin, 2011). Our preliminary field studies showed that the paddy fields in three regions along the river differed in soil physicochemical properties; therefore, paddy field soils were sampled from those three regions: Kizilirmak region, Osmancik region, and Samsun region. Since soil water is necessary for protists to be active, soil water content and climate are well-known factors to affect protist communities enormously (Geisen et al., 2018; Oliverio et al., 2020). In order to minimise their effects and to focus on the effects of soil properties and the rhizosphere effect of rice on protist communities, a soil microcosm study with and without rice plants was conducted in a growth chamber under the same conditions (soil water, temperature, humidity, rice variety etc.). This approach enabled us to directly evaluate how soil properties and the rhizosphere effect of rice shapes protist communities. The taxonomic diversity of the protist communities was characterised by a high-throughput sequencing method, and the taxonomic profiles were assigned to potential functionalities of protists.

2. Materials and methods

2.1. Soil samples and rice seedlings

The soil samples were collected from 11 paddy fields after the harvest across three regions along the Kizilirmak River in Turkey. Locations of the sampling sites and the chemical usage data were provided in Supplementary Figs. S1–2 and Table S1. The soil samples were collected in three consecutive days from the Kizilirmak (Sep 30, 2019), Osmancik (Oct 1, 2019), and Samsun (Oct 2, 2019) regions as follows: The surface layer (0–10 cm) was collected with a core sampler (10 cm depth, 5 cm radial) from randomly selected 15 sampling points in each field. Afterwards, the soil samples were air-dried, sieved (<2 mm), mixed thoroughly, and stored at 4 °C prior to further experiments. The numbers of fields were three for Kizilirmak and Osmancik regions and five for the Samsun region. The reason to obtain five samples from the Samsun region was that the pre-analysis of soil properties of the fields from the Samsun region showed higher variation among the samples. Each paddy field was represented with three replications. To evaluate the rhizosphere effect on protist community composition, we used *O. sativa* L. Osmancik97, the most common rice cultivar in Turkey. To minimise the transfer of seed-borne microorganisms to the microcosms, the rice seeds were sterilised with 0.2 M sodium hypochlorite and grown aseptically in a growth chamber as described previously (Asiloglu et al., 2020).

2.2. Experimental set-up and sampling

The microcosms were established in sterile 50 ml plastic tubes filled with the paddy field soils in six replications, giving a total of 66 microcosms. Half of the microcosms were planted with sterile rice seedlings (one seedling per microcosm). The microcosms were saturated with sterile H_2O. The rice plants were cultivated in a growth chamber (MLR-350, SANYO, Osaka, Japan) under submerged conditions at 25 °C with a day length of 15 h (250 μmol m^{-2} s^{-1}) for 21 days. The tubes were covered with aluminium foil except the top part to block the light penetration and were watered daily to maintain the submerged conditions. After 21 days of incubation, the microcosms were destructively sampled as previously described (Asiloglu et al., 2020). Briefly, the surface water was removed from the microcosms, and the soil was mixed thoroughly. A 0.5 g of soil sample was placed into 2 mL tubes and stored

at −80 °C until nucleic acid extraction.

2.3. Physicochemical analyses of soil

The pH was measured in deionised water at a 1:2.5 (w/v) mass ratio using a pH meter (FP20, Mettler Toledo, Greifensee, Switzerland), and the EC was measured using an EC meter (C3010, Consort, Turnhout, Belgium) as described by Rajkovich et al. (2012). The available phosphorus was estimated by extraction with sodium bicarbonate (Olsen, 1954). The soil organic matter (SOM) content was measured by the Walkley-Black method (Nelson and Sommers, 2015). Total N in the soil samples was determined with the Kjeldahl method (Bremner, 1965). The exchangeable cations (Ca, Mg, K, and Na) were extracted from the soil samples with 1 M ammonium acetate (pH 7.0) and measured in an atomic absorption spectrophotometer (McGeorge, 1954). Calcium carbonate content was measured using a constant volume calcimeter according to McGeorge's method (1954).

2.4. Molecular analysis and bioinformatics

DNA was extracted from 0.5 g of the soil samples using ISOIL for Bead Beating (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction and eluted in TE buffer (50 μL). The V9 region of the 18S rRNA gene was amplified from the extracted DNA using the universal eukaryotic primers (1389F/1510R) (Amaral-Zettler et al., 2009) tailed with the barcoded adapters (Illumina, San Diego, CA) (Caporaso et al., 2012). Primary polymerase chain reaction (PCR) was performed as described elsewhere (Amaral-Zettler et al., 2009). Purification of the PCR product, the Illumina MiSeq index PCR, and the Illumina MiSeq sequencing were performed as described previously (Asiloglu et al., 2020).

After sequencing, the primary analysis of raw FASTQ data was processed using the QIIME2 pipeline (version 2018.11, https://qiime2.org) (Bolyen et al., 2019)The DADA2 (Callahan et al., 2016) was used for error-correction, removal of forward and reverse primers, quality filtering, singleton and doubleton removal, and chimera removal of the Illumina amplicon sequences. The reads were truncated at 200 bp for each single-end read (corresponding to a quality score >30), which allowed forward and reverse sequences to overlap >50 bp. The QIIME2's q2-feature-classifier plugin was used for taxonomy assignment against the latest version (4.12.0) of the Protist Ribosomal Reference (PR^2) database (Guillou et al., 2012). To obtain exclusive protist data, non-protist sequences (*Fungi, Metazoa,* unidentified *Opisthokonta, Streptophyta, Rhodophyta*, and unclassified eukaryotes) were removed from all samples using the Qiime2 (taxa filter-table/seq). To compare protist communities between the treatments, sequence read numbers were normalized to the minimum sequence number (1 286) by random subsampling. Nonmetric multidimensional scaling (NMDS) analysis was performed to visualise the beta diversity dissimilarities based on Bray-Curtis distance matrix. To correlate the environmental parameters associated with the NMDS ordination, we used the *env* function in the vegan package of R program version 3.6.1 (https://www.r-project.org/).

Protist taxonomies were assigned into three functional groups (consumers, autotrophs, and pathogens) as described elsewhere (Singer et al., 2021). Functional subgroups were created for consumers (decomposers and predators) and pathogens (plant pathogens and animal/microbial parasites). Briefly, all organisms that used photosynthesis are assigned as autotrophs, while all organisms who uptake nutrients with phagocytosis were assigned as consumers, including microbial predators and decomposers. Any organisms reducing the fitness of their host are labelled as pathogens, including plant pathogens and microbial/animal parasites. Linear discriminant analysis effect size (LEfSe) (Segata et al., 2011) was performed using the Galaxy server (http://huttenhower.sph.harvard.edu/galaxy) in order to detect the specific bioindicator organisms. The detected bioindicator organisms are further assigned to more detailed functional groups. First, the organisms were assigned to the functional groups (consumers, autotrophs, and pathogens). Then subgroups were assigned as follows: Pathogens were divided into plant pathogens and animal/microbial parasites and consumers were divided into decomposers and predators. Since predators were the main bioindicator groups, we further assigned predators to known morphological groups (flagellates, amoeba [including amoeboflagellates], and ciliates).

Our results indicated a correlation between plant pathogens and phagotrophs. Since plant pathogens consist of mainly *Pythium* sp. (over 90%), we further investigated the interaction between *Pythium* sp. and the other protists. *Pythium* sp. is an important pathogen of rice roots, and their abundance in the rhizosphere is particularly important. Our results showed that the relative abundance of *Pythium* sp. in the rhizosphere samples was 10% on average, while it was 3% in the bulk soil. Therefore, the microbial co-occurrence network (Williams et al., 2014) for *Pythium* sp. was created for rhizosphere samples as follows: First, the microbial co-occurrence network between all pairs of protists at the genus level was evaluated using Spearman's correlation coefficient in the R program as described previously (Asiloglu et al., 2021b). Then, the significant positive ($r > 0.75$, $p < 0.05$) and negative ($r < -0.75$, $p < 0.05$) correlations between *Pythium* sp. and the other protists were screened out, and the co-occurrence network were visualised in the Cytoscape software v. 3.7.2 (Shannon, 2003).

2.5. Statistical analyses

All statistical analyses were performed using the R program unless otherwise specified (https://www.r-project.org/). The principal component analysis (PCA) was conducted to compare the overall physicochemical properties of the soils. The data were normalized using "scale = TRUE" option in the *prcomp* function, and the PCA analysis was conducted in R program version 3.6.1 (https://www.r-project.org/). Comparison between means was performed with Tukey's honestly significant difference (HSD) test at 0.05 level, which was carried out to detect significant differences among treatments for soil physicochemical properties, protist alpha diversity indices, and plant biomass using the R program with glht function in the multcomp package (Hothorn et al., 2008). The beta diversities were analysed with permutational multivariate analysis of variance (PERMANOVA) with 999 random permutations ($p < 0.05$) with the adonis function of the vegan package. Significant biological consistency and effect relevance of the specific protistan groups were analysed by LEfSe as follows: Firstly, the non-parametric factorial Kruskal-Wallis sum-rank test ($p < 0.05$) was conducted to detect features with significant differential abundances with one against all strategy of the multi-class analysis. After this step, linear discriminant analysis (LDA), in which the logarithmic score was set to 2.0, was conducted to estimate the effect size of each differentially abundant feature.

3. Results

3.1. Soil physicochemical properties

The soil samples obtained from each region exhibited differential physicochemical characteristics, and the regions were grouped separately by the PCA analysis (Fig. 1). Soil samples obtained from the Samsun region had relatively higher pH, nitrogen, and available phosphorus (Supplementary Table S1). Samples of the Kizilirmak and Osmancik regions had more similar soil physicochemical properties to each other than to the samples of the Samsun region (Fig. 1). The salt (Exchangeable Na and EC) and lime ($CaCO_3$ and exchangeable Ca) contents were higher in the Kizilirmak and Osmancik regions than in the Samsun region. CEC, exchangeable Mg, exchangeable K, SOM were slightly higher in the Osmancik region than the Kizilirmak region (Supplementary Table S1).

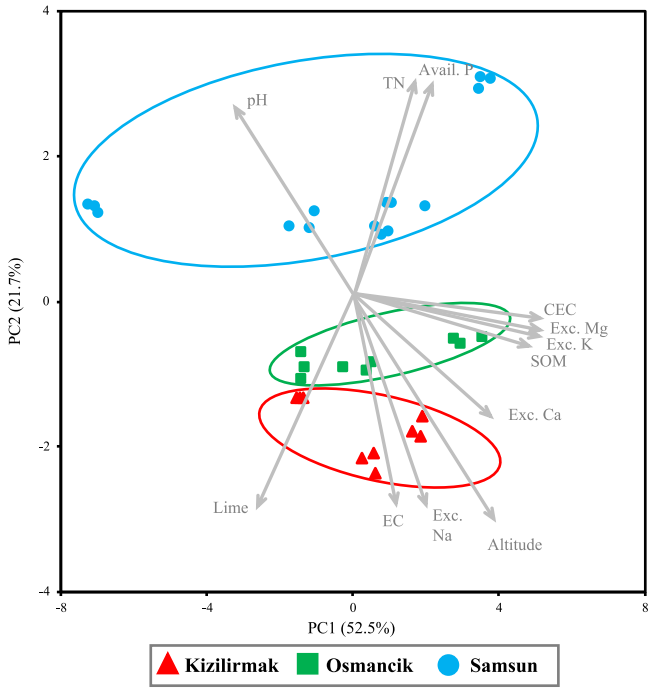

Fig. 1. Principal Component Analysis (PCA) based on the soil physicochemical properties. The soil samples were obtained in the Kizilirmak region (red triangle), Osmancik region (green square), and Samsun region (blue circle). Exc., exchangeable; avail., available. See Supplementary Table S1 for detailed information on the analysed soil properties. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

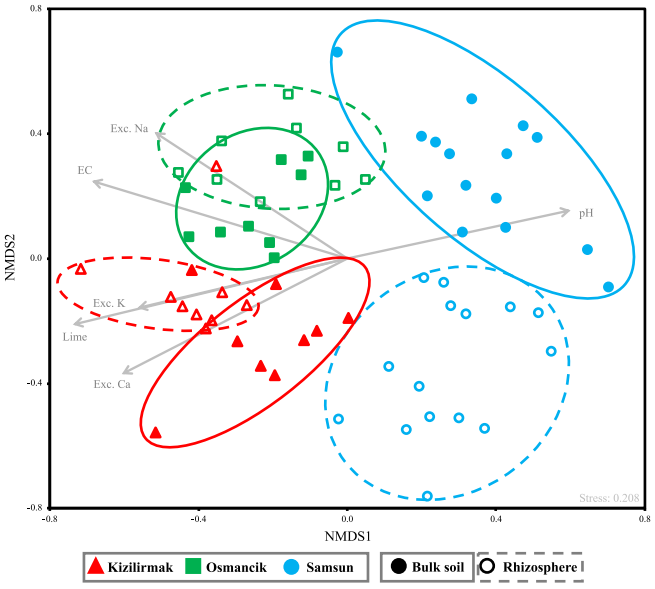

Fig. 2. Non-metric multidimensional scaling (NMDS) plots of protist communities with significant correlations ($p < 0.01$) with the soil physicochemical properties. Red triangles, Kizilirmak region; Green square, Osmancik region; Blue circle, Samsun region; Solid marks, Bulk soil; Hallow marks, Rhizosphere soil. The arrows indicate significant correlations among protist communities and environmental parameters. Exc., exchangeable. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Taxonomic community composition of protists

Overall, Amoebozoa were dominant (29.5%), followed by Stramenopiles (23.7%), Rhizaria (19.5%) and Alveolata (12.6%). The alpha diversity of protists and the richness (observed features) were not affected by the region nor the rhizosphere effect (Supplementary Fig. S3). The average number of OTUs with the standard deviation for each sample was 88 ± 22, ranged from 59 to 145. The differences in the soil physicochemical properties in each region were the major controlling factors on the beta diversity of protist communities (Table 1). The rhizosphere effect of rice significantly (R^2: 0.036, $p < 0.001$) affected the protist community composition. However, it was less prominent than the effects of the soil properties (R^2: 0.103, $p < 0.001$) (Table 1). The interaction between region and the rhizosphere effect was also significant (R^2: 0.058, $p < 0.001$) (Table 1). The NMDS analysis grouped the protist communities of each region (Fig. 2). The shift in the protist communities was significantly correlated with the soil physicochemical properties. Although the major factor grouping the protist communities together was the region effect, the rhizosphere effect of rice was separated the protist communities of the bulk and rhizosphere samples within each region (Fig. 2).

Overall, the rhizosphere effect of rice decreased the relative abundance of Amoebozoa and Rhizaria while increased the relative abundance of Stramenopiles and Alveolata (Fig. 3A). We observed a slightly different rhizosphere effect on protist taxonomic groups in each region (Supplementary Fig. S4). Comparison of the protist taxonomic groups of the regions (Fig. 3B) showed that the relative abundance of Amoebozoa increased from the Kizilirmak region to the Osmancik and Samsun regions. The relative abundance of Stramenopiles was lowest, and Alvaelota was highest in the Samsun region (Fig. 3B). Rhizaria was not affected by the regions, and the lowest relative abundance of Archaeplastida was observed in the Osmancik region (Fig. 3B).

Table 1
Permutational multivariate analysis of variance (PERMANOVA) results based on Bray-Curtis dissimilarities for the effects of region (soil properties) and plant roots.

Factors	Df	SumsOfSqs	MeanSqs	F. Model	R^2	p
Region	2	2.8495	1.4247	3.8307	0.1025	0.001 ***
Rhizosphere effect	1	1.0101	1.0101	2.7158	0.0363	0.001 ***
Region: Rhizosphere effect	2	1.6256	0.8128	2.1854	0.0585	0.001 ***
Residuals	60	22.3156	0.3719	0.8027		
Total	65	27.8008	1.0000			

3.3. Functional community composition of protists

The assignment of the taxonomic profiles to their respective functionalities (Fig. 3C and D) revealed that protist community compositions of different paddy field soils in the microcosms experiment were mainly dominated (67.7%) by the consumers (decomposers and predators), followed by the autotrophs (21.0%) and pathogens (microbial/animal parasites and plant pathogens) (9.2%). The 2.3% of the taxonomic groups were not assigned to any of the three functional groups. The rhizosphere effect significantly increased the pathogens while decreased the relative abundance of autotrophs and consumers (Fig. 3C). Different patterns of the rhizosphere effect on functional groups of protists were observed in each region (Supplementary Fig. S5). The relative abundances of consumers were decreased by the rhizosphere effect in both Kizilirmak and Osmancik regions, while it was not affected in the Samsun region. The pathogens were increased by the rhizosphere effect in all regions (Supplementary Fig. S5). A different profile of the functional groups was observed in each region (Fig. 3D). The samples obtained from the Samsun region showed the highest relative abundance of

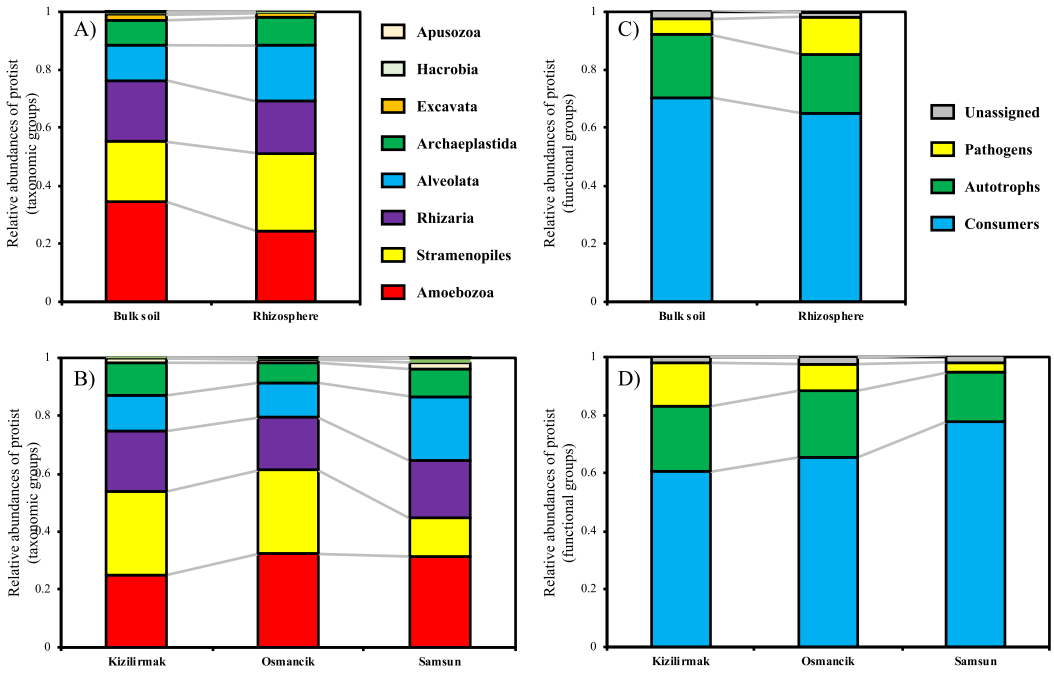

Fig. 3. Taxonomic (A and B) and functional (C and D) community compositions of protists affected by rhizosphere (A and C) and regions (B and D).

consumers and the lowest relative abundance of pathogens (Fig. 3D). The relative abundance of autotrophs and pathogens was highest in the Osmancik and Kizilirmak regions, respectively (Fig. 3D).

3.4. Bioindicator groups

The effects of different soil properties of each region and the rhizosphere effect on the protist community composition at multiple taxonomic levels were confirmed by the LEfSe analysis, which revealed the bioindicator protist groups in each region and those that reflect the rhizosphere effect (Fig. 4A and B). The bioindicator protists in the Samsun region mainly belonged to Alvaelota, Cercozoa, and Excavata (Fig. 4A). Lobosa and Pseudofungi were the main bioindicator groups in the Kizilirmak region, while the Osmancik region was characterised with the highest relative abundances of Breviatea and Centramoebida (Fig. 4A). The total number of the bioindicator groups was 25, 19 and 19 for Kizilirmak, Osmancik, and Samsun regions, respectively. The rhizosphere effect was clearly demonstrated by the LEfSe analysis, in which the total number of the bioindicator groups was 24 and 36 in the bulk soil and rhizosphere, respectively (Fig. 4B). Mainly Breviatea, Trebouxiophyceae, Preaxostyla, and Xanthophyceae characterised the bulk soil communities, while the rhizosphere communities were characterised by Heterobolosea, Discosea, Filosa, and Pseudofungi. The rhizosphere effect of rice showed slightly different patterns in each region (Supplementary Fig. 6).

The bioindicator protists were assigned to functional groups, which revealed that the majority of the bioindicator groups belonged to the subgroup of predators (phagotrophic protists) (Fig. 5). The soil bioindicators were dominated by the predators, mainly amoeba and flagellates in the bulk soil, while the rhizosphere was dominated by amoeba and ciliates (Fig. 5A). Among the subgroups of pathogens, microbial and animal parasites were detected in the bulk soil as bioindicators, and plant pathogens were detected in the rhizosphere (Fig. 5A). Although predators were the dominant bioindicator groups in all regions, each region was characterised by different predators: Amoeba and flagellates in the Kizilirmak region, only amoeba in the Osmancik region, and ciliates and flagellates in the Samsun region (Fig. 5B). No pathogen-related bioindicator group was detected in the Samsun region, while the Osmancik region contained microbial and animal parasites and the Kizilirmak region contained both microbial and animal parasites and plant pathogens (Fig. 5B).

We found that the relative abundance of *Pythium* sp. was negatively correlated (Regression analysis, $R^2 = 0.4126$, $p = 0.00005$) with the relative abundance of phagotrophs in the rhizosphere samples (Fig. 6A). The network analysis shows the interaction between *Pythium* sp. and other protists (Fig. 6B). We found that over 75% of the interactions between phagotrophs (predators) and *Pythium* sp. were negative ($r < -0.75$). Among them, the negative interaction with amoeba (Mastigamoebidae) was most strongly observed (Fig. 6B). We observed mainly positive correlations between autotrophs and *Phytium* sp., indicating that they may have similar preferences.

3.5. Growth of rice plants

The rice plants exhibited healthy growth in all microcosms. The rice plants grew in the soil samples obtained from the Samsun region showed significantly higher shoot biomass ($p < 0.05$) than those grown in the soil samples of the Kizilirmak and Osmancik regions (Supplementary Fig. S7). The average shoot biomass in the Samsun, Osmancik and Kizilirmak microcosms was 0.143, 0.123 and 0.126 g, respectively.

4. Discussion

Here, we provided a unique overview of protist communities in alkaline paddy field soils, where consumers, in particular phagotrophic protists, were the most abundant protist group. Both the soil properties and the rhizosphere effect of rice significantly affected the community composition of protists in alkaline paddy field soils, which agreed with our hypothesis. We found that soil properties have a bigger impact on protist community composition than that of the rhizosphere effect of rice. In this study, we did not analyse the protist community directly in the paddy field but instead examined the protist community after establishing a microcosms study to achieve the purpose of the study. Therefore, it should be noted that the results obtained in this study indicate the potentially similar protist communities with those of the actual paddy field environments. During the sampling period, we

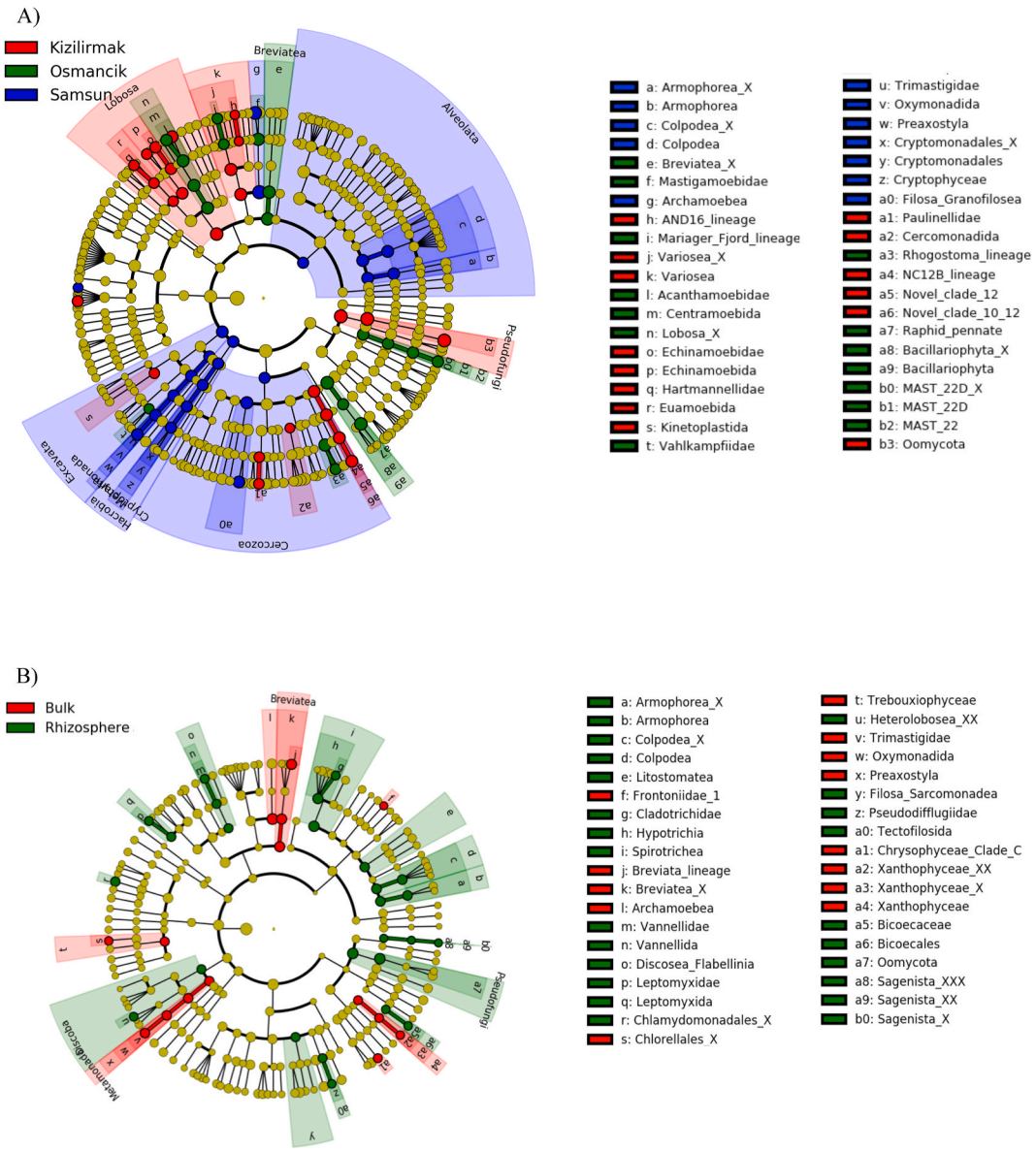

Fig. 4. A linear discriminant analysis effect size (LEfSe) identifies the significantly different ($p < 0.05$, Kruskal–Wallis test, LDA score >2.0) protists at multiple taxonomic levels by comparison of protist communities in the three regions (A) and in the rhizosphere and bulk soils (B). Cladograms are illustrating the taxonomic groups that explain the most variation among protist communities. Coloured dots represent the taxa with significantly different abundances between treatments, and from the centre outward, they represent the kingdom, phylum, class, order, family, and genus levels. The coloured shadows represent trends of the significantly differed taxa. A) Red colour, Kizilirmak region; Green colour, Osmancik region; Blue colour, Samsun region. B) Red colour, bulk soil; green colour, rhizosphere. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

observed significant differences in soil water content due to climate and soil management. It is well known that differences in soil water content due to climate and soil management at the sampling can significantly affect the local protist communities (Geisen et al., 2014, 2018; Murase et al., 2014). Soil water content is likely to affect the taxonomic and functional diversity of protists before other factors such as soil nutrients and the rhizosphere effect, masking the effects of the other factors on protist diversity (Geisen et al., 2014; Murase et al., 2014). Therefore, we believe that our approach to minimising the effect of water and climate allowed us to compare the soil physicochemical properties and the rhizosphere effect on the protist diversity in submerged paddy soils.

4.1. *Effects of soil properties on protist community composition*

The alkaline paddy field soil in the microcosms was dominated by consumers (predators and decomposers), which is in line with the protist communities in acidic paddy field soils (Asiloglu et al., 2015; Murase et al., 2015; Asiloglu and Murase, 2016) as well as overall global soils (Oliverio et al., 2020). The visualisation of protist beta diversity by NMDS analysis (Fig. 2) showed similar patterns with the PCA analysis based on the soil properties (Fig. 1), indicating the importance of soil properties controlling protist communities. The soil pH was significantly correlated with the shifts in the protist community composition. Amoeba was the main bioindicator group in Kizilirmak and Osmancik regions, while the main bioindicator groups were ciliates in the Samsun region, where the soil pH was highest. Previously, Shalinimol et al. (2009) showed that the number of ciliates was decreased in paddy field soil with lower pH values. This is in line with the results of a global scale distribution of soil protists, where ciliates (*Ciliophora, Alveolata*) were most abundant in soils with relatively higher pH values (Dupont et al., 2016; Oliverio et al., 2020). Although more evaluations are needed, our results indicate that the pH of paddy field soil may be an important

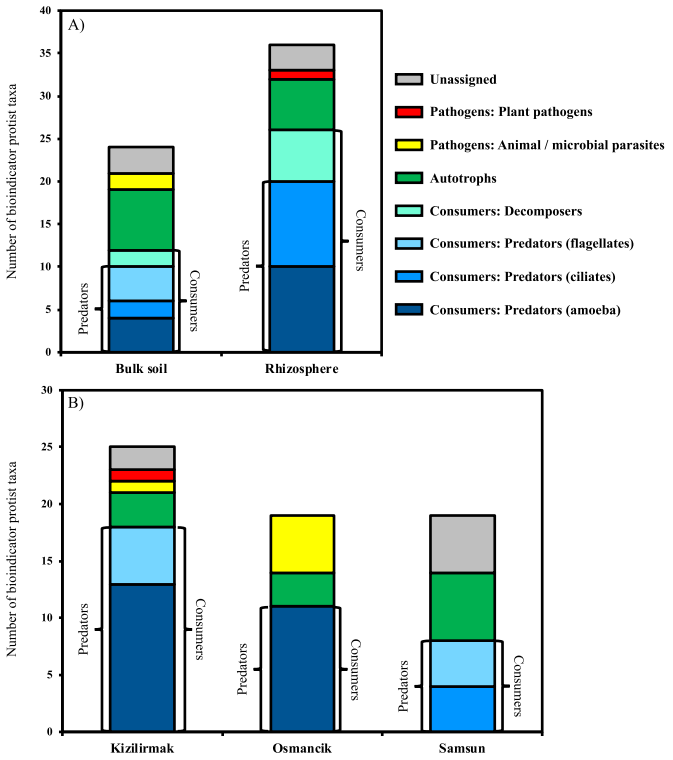

Fig. 5. Functional assignment of the bioindicator protist taxa identified by LEfSE. Comparison of the protist communities in the bulk soil and the rhizosphere (A) and the regions (B). The bar plot shows the total number of the identified bioindicators.

factor controlling the diversity of predatory protists, especially ciliates.

Nitrogen is one of the important factors strongly affecting protist communities (Puigagut et al., 2005; Angus et al., 2014; Zhao et al., 2019, 2020). We did not find a correlation between soil nitrogen content and the shift in the protist communities, probably due to the high similarities in nitrogen content of soils between the treatments. Soil salinity is a major problem in Turkey, especially in the Central Anatolian region (Gorji et al., 2017). Our results showed that soil salinity (EC), which was significantly higher in the Osmancik and Kizilirmak regions than that of the Samsun region, was correlated with the shift in the protist community composition. Although the importance of salinity for protist communities is well-known for marine and freshwater environments (Logares et al., 2009; Balzano et al., 2015; Singer et al., 2021), to the best of our knowledge, there is no similar report on the interaction between soil salinity and protist communities. Considering the importance of protists for soil health (Gao et al., 2019; Xiong et al., 2020), how soil salinity influences protist communities should be further investigated.

The relative abundance of the parasites decreased almost three-fold in the Samsun region compared to the Osmancik and Kizilirmak regions. This decrease was correlated with the soil physicochemical properties in our study. On the other hand, the abundances of animal/microbial parasites depend on animal diversity—for instance, arthropods—in soil, rather than the direct influence of soil properties (Mahé et al., 2017). Since no previous data is available on animal diversity of the studied paddy fields, we cannot conclude whether the decrease in the relative abundance of the parasites is related to the differences in the soil physicochemical properties or not.

4.2. The rhizosphere effect of rice plant on protist community composition

The LEfSe analysis indicated that the rhizosphere effect of rice mainly altered the community composition of phagotrophic protists (Fig. 5A), which is in line with the previous studies (Asiloglu et al., 2015;

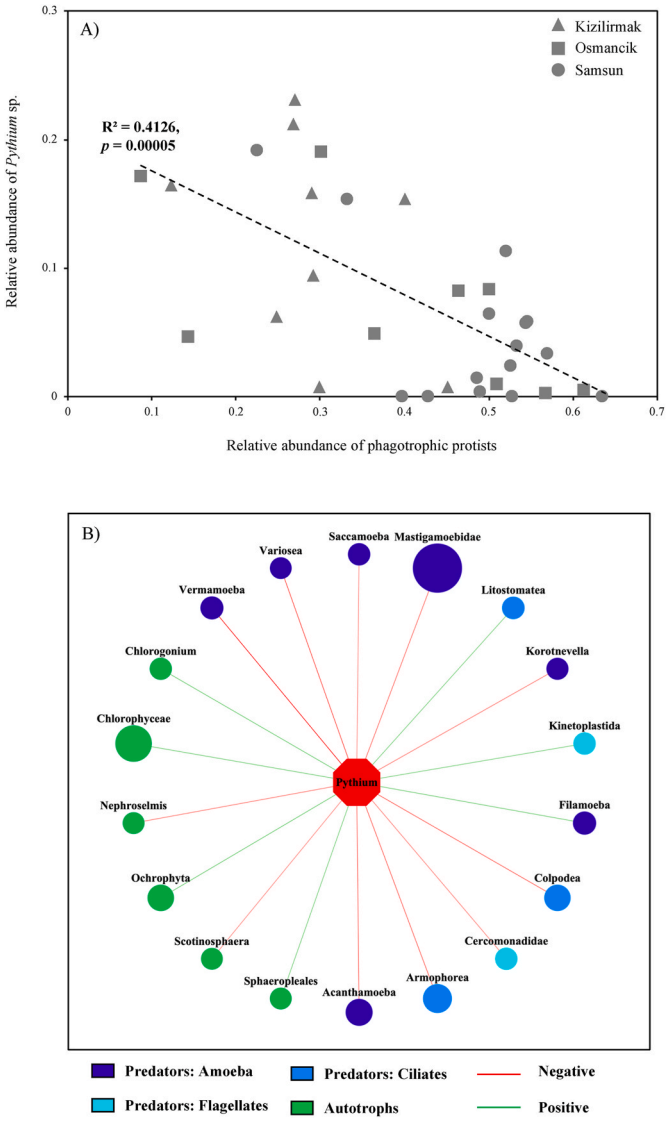

Fig. 6. Interaction between the plant pathogen (*Pythium* sp.) and phagotrophic protists. A) The regression analysis showing the significant correlations between the relative abundance of phagotrophic protists and *Pythium* sp. in the rhizosphere of rice plants. B) Microbial co-occurrence network analysis of the protists that are directly associated with the *Pythium* sp. in all samples of the rice rhizosphere. The network analysis was conducted with the taxonomic data of the protists, and then the taxonomies were assigned to the functional groups (Predators, phagotrophic protists; Dark blue colour, amoeba; blue colour, ciliates; light blue colour, flagellates. Positive co-occurrence correlations (Spearman's $r > 0.75$, $p < 0.05$) were indicated with green-coloured edges, while negative co-occurrence correlations (Spearman's $r < -0.75$, $p < 0.05$) were indicated with red-coloured edges. The node sizes indicate the mean taxonomic abundances. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Asiloglu and Murase, 2016). The rhizosphere effect of rice occurs due to the root exudates and rhizodeposition, which directly affects the microbial communities using the root-derived organic matter (Kimura et al., 1979). However, this does not apply to protists, except a few saprotrophs and pathogens, as the majority of protists do not live on nutrients but microbial prey (Geisen et al., 2018). Thus, the rhizosphere effect via the exudates and rhizodeposition may not likely have direct effects on protists. Previously Zhang et al. (2017) showed that the community composition of phagotrophic protists was driven by communities of bacterial prey. Bacterial communities differ in the rhizosphere and bulk soil of paddy field (Li et al., 2019) and rice roots

enhance bacterial populations (Kimura et al., 1979; Kirk, 2004; Hussain et al., 2012). Although we don't have direct evidence in this study, the rhizosphere effect of rice on the phagotrophic protist community may be indirect through altering the prey communities and enhancing prey populations, which should be further investigated. On the other hand, the rice roots in the early growth stage release oxygen to the surrounding soil making the rhizosphere oxic (Gotō and Tai, 1956). Murase et al. (2014) showed that oxygen is one of the primary factors affecting protist community composition, mainly phagotrophs, in a paddy field soil. Therefore, we propose that the rhizosphere effect of rice may shape the phagotrophic protist communities through both direct (oxygen supply) and indirect (altering prey communities) effects.

The relative abundance of the plant pathogens, mainly *Pythium* sp., was increased in the rice rhizosphere compared to the bulk soil. *Pythium* members are commonly present in the soil or water in a paddy field and attach to plant roots, especially in the early plant growth stage (Chun and Schneider, 1998), and cause enormous yield loss (Kumar et al., 2009). A negative correlation was observed between *Pythium* sp. and phagotrophs in this study (Fig. 6A). The network analysis also indicated a negative interaction between *Pythium* sp. and phagotrophs (Fig. 6B). Although our results only indicate a correlation, a recent study with tomato plants (Xiong et al., 2020) showed a significant negative correlation between the abundance of phagotrophs and plant pathogens, including *Phytium* sp., suggesting that predator-prey interactions may affect the abundance of the plant pathogens. Some of the phagotrophic protists feed plant pathogenic bacteria and fungi (Geisen et al., 2016; Gao et al., 2019); however, there is no evidence that *Pythium* sp. is a preferred food for phagotrophs. On the other hand, the negative correlation between phagotrophs and plant pathogens may be better explained by the indirect mechanism: the presence of phagotrophs affecting the production of bacterial secondary metabolites (Jousset et al., 2006; Amacker et al., 2020), which are highly effective components to suppress plant pathogens including *Pythium* sp. (Buysens et al., 1996; Hultberg et al., 2010). So far, only a handful of studies have investigated the interaction between phagotrophs and plant pathogens (Geisen et al., 2016; Long et al., 2018; Gao et al., 2019; Xiong et al., 2020). A better understanding of trophic regulation of plant pathogens may contribute to rice plant health.

5. Conclusion

Here, we revealed that the protist communities were driven by the soil physicochemical properties and the rhizosphere effect of rice roots in alkaline paddy field soils of Turkey. We showed that the soil physicochemical properties have a bigger effect determining protist communities than those of the rice roots. Our finding further showed that phagotrophic protists, the predators, are the dominant protist groups in the bulk and rhizosphere soils of alkaline paddy fields. As protist communities, especially phagotrophs, were differed depending on the soil properties, they may have different the top-down control on bacterial communities in soil food-web of paddy fields. In addition, we showed a significant negative correlation between phagotrophic protists and plant pathogens, which indicates that the plant pathogens could be top-down controlled by the phagotrophs. Further research on the whole microbiome (not only protists but also bacterial, archaeal, and fungal communities) should provide a better picture of the microbial food-web in paddy field soil.

Authors' contributions

RA, NH, OCT, and K Suzuki conceived and designed the study. RA performed bioinformatics and statistical analyses, interpreted the data, and prepared the manuscript. K Shiroishi and OCT performed sampling. K Shiroishi conducted the experiments. All authors provided feedback, read, and approved the final manuscript.

Data availability

The raw sequence data obtained in this study have been deposited in the NCBI database under the BioProject ID PRJNA728817.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2021.108397.

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