Evaluation of seasonal dynamics of fungal DNA assemblages in a flow-regulated stream in a restored forest using eDNA metabarcoding

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Abstract

Investigation of seasonal variations in fungal communities is essential for understanding biodiversity and ecosystem functions. However, the conventional sampling method, with substrate removal and high spatial heterogeneity of community compositions, makes surveying the seasonality of fungal communities challenging. Recently, water environmental DNA (eDNA) analysis has been explored for its utility in biodiversity surveys. In this study, we assessed whether the seasonality of fungal communities can be detected by monitoring eDNA in a forest stream. We conducted monthly water sampling in a forest stream over two years and used DNA metabarcoding to identify fungal eDNA. The stream water appeared to contain functionally diverse DNA from both aquatic and terrestrial fungi, such as plant decomposers, parasites, and mutualists. The DNA assemblages showed a distinct annual periodicity, meaning that the assemblages were similar to each other regardless of the year in the same sampling season. Furthermore, the strength of the one-year periodicity may vary across functional groups. Our results suggest that forest streams act as a "natural trap" for fungal DNA, and that studies of fungal DNA in stream water may provide information about the temporal variations in fungal communities inhabiting not only water but also the surrounding terrestrial ecosystem.

Keywords:

biodiversity, environmental DNA, fungal community, metabarcoding, seasonality, time-series data

Introduction

Fungi include diverse functional groups, such as decomposers, symbionts, and parasites, and thus play an important role in regulating ecosystem functions by driving the material cycling of ecosystems and influencing the population dynamics of other organisms (Peay et al., 2016; Grossart et al., 2019). As the functional capacities of fungi may often differ among species (Nara, 2006; Osono, 2020; Zanne et al., 2020), it is essential to explore the spatiotemporal patterns of fungal communities, and the factors that shape them, to understand and predict how fungus-driven ecosystem functions respond to environmental changes (Lilleskov and Parrent, 2007). Previous studies have shown the spatial and temporal turnover of fungal communities in a variety of substrates, such as soils, living/dead plant tissues, and animal digestive tracts. For example, the species composition of fungi varies at spatial scales of a few tens of centimeters to several meters (Bahram et al., 2016; Peay et al., 2016) or at time scales of several months (Voříšková et al., 2013; Matsuoka et al., 2016; Sugiyama et al., 2020). In particular, the recent proliferation of high-throughput sequencing techniques has facilitated the simultaneous processing of a large number of samples in parallel, and information about the spatial variations of terrestrial and aquatic fungi in various ecosystems is rapidly accumulating (Peay et al., 2016; Grossart et al., 2019; Nilsson et al., 2019). In contrast, studies on temporal variations have been far less frequent.

Typical factors causing temporal variation in the community include seasonal fluctuations in climate (e.g., temperature) and host phenology. Although studies on temporal variations in terrestrial and aquatic fungal communities are limited, several reports have demonstrated seasonal changes in community composition and the occurrence of fruiting bodies (Sato et al., 2012; Voříšková et al., 2013; Taylor and Cunliffe, 2016). For example, saprotrophic fungi associated with tree leaves show seasonal patterns in response to the supply of tree foliage as a substrate (e.g., Voříšková et al., 2013). If the occurrence of fungal species and their associated fungal functions change seasonally, a one-time community survey (i.e., snapshot) cannot capture the fungal diversity and function at a study site and may lead to misestimations of diversity and function. For example, if the fungal community shows changes in response to seasonal defoliation events, the community composition may show an annual pattern, such as a one-year periodicity. To detect such seasonal patterns, a continuous, multi-year monitoring study is needed (Bahram et al., 2014; Matsuoka et al., 2016; Sugiyama et al., 2020); however, as these surveys are limited, they hinder our understanding of the fungal community seasonality.

Investigating seasonal variations in fungal diversity based on time series sampling requires ingenuity in sampling, particularly in terrestrial habitats. Fungal communities in terrestrial habitats have been surveyed by collecting substrates from the field (Lindahl et al., 2013; Osono, 2014). Destructive sampling (i.e., a collected substrate is permanently lost from a study site) is a common method for investigating fungal community structure. Previous studies have shown that the fungal community composition in soils and plant substrates exhibits a high degree of spatial heterogeneity, where turnover can occur at scales of tens of centimeters to several meters (Bahram et al., 2016; Peay et al., 2016). Such compositional turnover is caused not only

by environmental heterogeneity (e.g., soil chemistry and host plants) but also by stochastic factors, such as dispersal limitation and drift (Bahram et al., 2016; Peay et al., 2016). The large spatial turnover in community composition means that, to reflect the entire community, multiple samples must be taken from different places to represent the fungal community structure at the site. The collection and processing of many samples are not only expensive and time- and labor-consuming but can also cause disturbances in the form of substrate removal from the study area. Therefore, time series sampling may need to be designed to address these challenges.

Aquatic environmental DNA (eDNA) analysis may be used to overcome these difficulties. In recent years, the utility of aquatic eDNA surveys in biodiversity research has been explored, as this method allows for detection of the DNA of organisms not only in water but also in surrounding terrestrial areas (Deiner et al., 2016; Khomich et al., 2017; Matsuoka et al., 2019). For example, Matsuoka et al. (2019) reported that the DNA of both aquatic and terrestrial fungi can be detected in river water in a forest landscape. This may be because the river water contains spores and mycelial fragments of fungi that have entered it from the surrounding terrestrial areas (Voronin, 2014). Furthermore, the fungal DNA assemblages are spatially structured; that is, similar DNA assemblages are found between rivers that are geographically close to each other and within a single tributary, but not between other tributaries (Matsuoka et al., 2019). This suggests that the investigation of fungal DNA assemblages, including those of both aquatic and terrestrial fungi, in river water may provide information on the diversity in the catchment area. Owing to these characteristics of aquatic eDNA, using these surveys in river and stream water may be potentially useful for addressing the above-mentioned challenges. First, water sampling does not involve the removal and destruction of terrestrial substrates such as soils; therefore, disturbance to an ecosystem is minor compared with that caused by conventional survey methods. Second, spatial heterogeneity is likely to be much lower than that in terrestrial substrates because of the high mobility of stream water. Indeed, the usefulness of aquatic eDNA in capturing seasonal community dynamics has been confirmed in aquatic invertebrates (Bista et al., 2017). However, the analysis of fungal DNA assemblages in water has only recently begun, and little is known regarding the seasonal patterns exhibited by the assemblages.

The objectives of the present study were to investigate whether seasonal patterns of fungi can be detected through time series sampling of forest stream water samples and fungal eDNA analysis. In particular, we addressed the following questions: (1) Which fungal DNA is detected in the forest stream water? (2) Do fungal DNA assemblages in water show temporal variations, particularly in seasonal patterns? (3) If the DNA assemblages show seasonal variation, is the variation related to climatic factors? This study was conducted in a flow-regulated stream within a restored forest fragment in an urban setting, with a particular focus on seasonal temporal variation. Here, the stream flow was controlled to maintain it within a certain range, depending on the amount of rainfall. In addition, unlike those in natural forest ecosystems, there were few large-scale disturbances, such as flooding. These settings allowed us to focus on seasonal changes, with minimal effects due to disturbances. Continuous water sampling was conducted once a month for two years. Soil and plant tissues were also sampled at the study site to estimate the

origin of fungal DNA detected in the stream water, although this was preliminary. The fungal assemblages in the water, soil, and plant samples were analyzed via DNA metabarcoding. We identified taxonomic and functional groups by checking the database and evaluating the temporal patterns.

Materials and methods

Study site and sampling

The study site is a forest fragment located in Kyoto, in the western part of Japan (34.986751 ° N, 135.744950 ° E). It was a 30-year-old secondary growth forest (Fig. S1, approximately 0.6 ha) dominated by evergreen trees (Quercus glauca and Castanopsis sieboldii) and deciduous oak trees (Quercus serrata), which are the main natural vegetation in the area. A small stream runs through the forest and drains the groundwater. The stream covers an area of 592 m2, and the flow is controlled to be approximately 0.5 L per second. Rainfall is another source of stream water. When rainfall is high, groundwater pumping is reduced to regulate flow. The mean annual air temperature is 16.3 °C, and the mean annual precipitation is 1608.4 mm according to the Automatic Metrological Data Acquisition System (AMeDAS, Japan Meteorological Agency) at Kyoto Station, located 3 km northwest of the study site. This system automatically measures air temperature, precipitation, and solar radiation on the ground and has been established at approximately 1,300 locations throughout Japan. The forest fragment is used as a biotope and is located more than 3 km away from the surrounding forests.

From December 2016 to November 2018, 1 L of surface water was collected using bleached plastic bottles once a month from each of the three sampling points along the stream (Fig. S1). The ammonium compound benzalkonium chloride (1 mL) was added in situ to prevent DNA degradation (Yamanaka et al., 2017). The bottles were stored in a cool, dark place and brought back to the laboratory for immediate filtration. The water samples and controls were vacuum-filtered through 47 mm GF/F glass filters (pore size 0.7 μm, GE Healthcare, Little Chalfont, UK). The filters were stored at −20 °C before DNA extraction. Total DNA was extracted from the filters using a PowerSoil Kit (Qiagen, Hilden, Germany). First, in a 2 mL tube, each filter was cut into small pieces using bleached dissecting scissors. Thereafter, the chopped filters were placed in bead tubes provided with the kit and vortexed for 20 min. DNA were then extracted according to the manufacturer's instructions.

We calculated the mean daily air temperature (T), cumulative precipitation (P), and total solar radiation hours (S) for three days (3 d) and two weeks (2 w) prior to the survey date. Data on air temperature, rainfall, and solar radiation during the study period were obtained from the AMeDAS Kyoto station (see above). The mean daily temperatures for 3 d and 2 w were expressed as T3d and T2w, respectively. These cumulative durations (3 d and 2 w) were selected based on previous studies and fungal life history (Matsuoka et al., 2016). We did not perform a comprehensive search of optimal cumulative durations to avoid high computational and runtime

costs. The climatic variables at each sampling site are listed in Table S1.

In addition, to determine potential sources of fungal DNA detected in the stream water, we conducted additional soil and plant tissue (fresh and fallen leaves) sampling in the study forest in February 2021. First, one soil block of the fermentation-humus layer (10 cm × 10 cm each, approximately 5 cm depth), excluding the surface litter, was collected from each of the 10 sites. Three fresh and fallen leaves of two dominant evergreen species (Q. glauca and C. sieboldii) were collected from three sites. The collection sites are shown in Fig. S1. The collected samples were immediately brought back to the laboratory in cold bags for DNA extraction. In the laboratory, 250 mg of each soil sample was weighed out. The fresh leaves and fallen leaves were first rinsed and wiped with sterile water to remove particles adhering to the leaf surfaces. Then a 5 mm square piece of tissue was then cut from each leaf and combined at each site. DNA was extracted from a total of 22 samples: 10 soil and 12 plant tissue samples (2 tree species × 2 types (fresh or fallen) × 3 sites). For comparison with the stream water samples, DNA was extracted using the same procedure as for the water samples using the PowerSoil Kit.

Molecular identification of fungi

The procedures used for molecular experiments and bioinformatics were those described previously (Matsuoka et al., 2019). In brief, the fungal internal transcribed spacer 1 (ITS 1) region of rDNA was amplified using the ITS1-F-KYO2 (5′- TAG AGG AAG TAA AAG TCG TAA -3′) and ITS2-KYO2 (5′- TAG AGG AAG TAA AAG TCG TAA -3′) primer sets (Toju et al., 2012). The PCR amplicons were pooled and sequenced using the Illumina MiSeq platform at the Center for Ecological Research, Kyoto University, Japan. The sequence data were deposited in the Sequence Read Archive of the DNA Data Bank of Japan (accession number: DRA011182 and DRA012017). See Appendix 1 for details of the molecular analyses.

The raw MiSeq data were converted into FASTQ files using Bcl2gastq v2.18.0.12 and then demultiplexed using Claident pipeline (Tanabe and Toju, 2013; software available online: https://www.claident.org/). For the forward and reverse sequences, those with overlaps were concatenated using the default settings of VSEARCH. If the sequence contained more than 10% of bases with qualities less than 30, the sequence was excluded. A total of 2,169,706 reads (30,135 ± 12,751 reads per sample, mean ± SE, n = 72) from water samples and 1,022,977 reads (46,499 ± 10,634 reads per sample, mean ± SE, n = 12) from soil and plant samples were assembled using Claident v0.2.2018.05.29. Potential chimeric sequences and sequencing errors were removed using UCHIME v4.2.40 (Edger et al., 2011) and algorithms in CD-HIT-OTU (Li et al., 2012), respectively. The remaining sequences were assembled at a threshold similarity of 97% (Osono, 2014), and the resulting consensus sequences represented the molecular operational taxonomic units (OTUs). For each of the obtained OTUs, taxonomic identification was conducted based on the query-centric auto-k-nearest-neighbor method (Tanabe and Toju, 2013) with the NCBI database, and subsequent taxonomic assignment was conducted using the lowest common ancestor algorithm (Huson et al., 2007) using Claident. The functional guild of each fungal OTU

was estimated based on the FUNGuild database (Nguyen et al., 2016). One hundred OTUs (48,685 reads) that were identified as non-fungal organisms were discarded.

Data analysis

The bioinformatic pipeline described above generated an OTU table (i.e., matrix of OTUs and samples with sequence reads in each cell entry). For this OTU matrix, cell entries with reads less than 0.0002% of the total reads in each sample (which corresponds to 2–13 reads, a typically used criterion in eDNA metabarcoding studies, Table S2) were removed because these rare entries could represent contamination. After this process, 1,935,576 reads and 4,388 OTUs were obtained. The number of sequences, taxa, functional groups, and consensus sequences of the obtained OTUs is shown in Table S2. All analyses were performed using R v.3.4.3 (R Core Team 2017). We confirmed that the number of sequence reads was enough to detect OTUs in the samples using the 'rarefy' and 'rarecurve' functions of the 'vegan' package (Fig. S2). Thus, we used raw data for further analyses without rarefying the data. The presence or absence of OTUs was recorded. Binary data were used for all the statistical analyses.

To test whether OTU compositions differed across sampling occasions and three sampling points, we conducted permutational multivariate analysis of variance (PERMANOVA), using the 'adonis2' command in the 'vegan' package ver. 2.5-6 with 9999 permutations. The presence/absence of the OTU data for each sample (n = 72) was converted into a dissimilarity matrix using the Raup-Crick index with 9999 permutations. The Raup-Crick dissimilarity index is calculated based on the null model approach, which is akin to the standardized effect size (SES) value described below. The community dissimilarity matrix was visualized via nonmetric multidimensional scaling (NMDS) using 'ordinate' and 'plot_ordination' commands in 'phyloseq' package ver. 1.28.0.

Subsequent analyses were performed for all OTUs and for each functional group (i.e., saprotrophic, symbiotic, and parasitic fungi). To evaluate temporal variations in OTU compositions among sampling occasions, the presence/absence data for each sampling occasion were merged. First, the effects of sampling year (first or second year) and month (i.e., twelve categories) on the OTU composition were tested with PERMANOVA ('adonis2' command, 9999 permutations). The presence/absence of the OTU data for each sampling occasion (n = 24) was converted into a Raup-Crick dissimilarity matrix with 9999 permutations. Next, the temporal dynamics of the OTU compositions were tested. The OTU matrix was converted into a dissimilarity matrix using the SES of the Jaccard dissimilarity index. The SES was defined as (Disobs–Disnull)/Dissd, where Disobs is the observed dissimilarity, Disnull is the mean of the null distribution of dissimilarity, and Dissd is the standard deviation of the null distribution. The null distribution was calculated based on 9999 randomizations, preserving both the site of occurrence and the OTU richness with 'randomizeMatrix' command in the 'picante' package. Thereafter, the dissimilarity matrix was visualized using NMDS. Temporal changes in SES values were tested by comparing SES values and temporal distances via generalized additive models (GAMs) using the 'gam' command in the 'mgcv' package ver. 1.8-31.

Finally, to estimate the contribution of climatic and temporal factors to the temporal changes in OTU composition, variation partitioning based on a distance-based redundancy analysis was conducted using the 'capscale' command in the 'vegan' package. The relative weight of each fraction (pure and shared fractions and unexplained fractions) was estimated using the methodology described by Peres-Neto et al. (2006). The Raup-Crick dissimilarity matrix was used for each sampling occasion. The detailed methods for variation partitioning are described in Matsuoka et al. (2016). Briefly, we constructed two models, including climatic and temporal distance variables, by applying the forward selection procedure (999 permutations with an alpha criterion = 0.05) of Blanchet et al. (2008). The full climatic model included six climatic variables (i.e., T3d + T2w + P3d + P2w + S3d + S2w). We then constructed a model using temporal distance vectors calculated using Moran's eigenvector maps (MEM, Borcard et al. 2004). The MEM analysis produced a set of orthogonal variables derived from a temporal distance matrix, and MEM vectors represented various temporal patterns, such as periodicity. First, we created a temporal distance matrix between each sampling occasion based on the sampling dates. Next, MEM vectors were calculated from the temporal distance matrix using the 'dbmem' command in the 'adespatial' package. We used the 11 MEM vectors that best accounted for autocorrelation and then conducted forward selection (Table S1). Based on these two models, variation partitioning was performed by calculating the adjusted R2 values (Peres-Neto et al., 2006).

Results

Fungal diversity in the stream water

Seventy-two water samples were obtained through a two-year (24 times) survey from 2016–2018. A total of 1,935,576 reads from 72 samples were grouped into 4,409 operational taxonomic units (OTUs) with 97% sequence similarity (Table S2). The number of OTUs per sample was 350 ± 109 (mean ± standard error [SE]). In total, 1,683 OTUs were assigned as Ascomycota (38.2% of the total number of fungal OTUs), 734 as Basidiomycota (16.7%), 68 as Chytridiomycota (1.5%), 27 as Mucoromycota, two as Blastocladiomycota, and one OTU was assigned as Entorrhizomycota (Fig. S3). The remaining 1,894 OTUs were not assigned to any phylum. At the order level, 1,112 Ascomycota OTUs belonged to 46 orders, 541 Basidiomycota OTUs belonged to 32 orders (Fig. S3), and the remaining OTUs were not assigned at the order level. The OTUs of the common ascomycete orders were Pleosporales (205 OTUs), Hypocreales (165 OTUs), Capnodiales (92 OTUs), Xylariales (81 OTUs), and Helotiales (79 OTUs). OTU numbers of the common basidiomycete orders were Agaricales (142 OTUs), Polyporales (77 OTUs), Corticiales (44 OTUs), Tremellales (38 OTUs), and Russulales (31 OTUs) (Fig. S3). At the genus level, 536 Ascomycota OTUs were assigned to 246 genera and 381 Basidiomycota OTUs to 176 genera; the remaining OTUs were not assigned at the genus level. The OTU-rich genera of ascomycetes and basidiomycetes were Penicillium (19 OTUs), Colletotrichum (16 OTUs), Fusarium (11 OTUs),

Cortinarius (14 OTUs), Tomentella (14 OTUs), and Trechispora (12 OTUs). Detailed taxonomic assignments are presented in Table S2.

FUNGuild assigned 1,250 OTUs to functional guilds, 609 OTUs of which were saprotrophs; the others included parasites (261 OTUs) and symbionts (111 OTUs) (Table S2, Fig. S4). The remaining 269 OTUs included endophytes and OTUs with multiple functions. The major saprotrophs were plant saprotrophs (205 OTUs), including those considered to be aquatic hyphomycetes (Alatospora and Tetracladium) and terrestrial wood and leaf saprotrophs (e.g., Ganoderma and Mycena). The major parasites were plant pathogens (192 OTUs), such as Taphrina and Ciboria. Among the symbionts, the major fungi were ectomycorrhizal fungi (91 OTUs), such as Cortinarius and Russula. These plant parasites and ectomycorrhizal fungi were terrestrial fungi, validating our expectation that terrestrial eDNA could be detected in stream water.

Temporal patterns of fungal OTU compositions and related variables

A total of 72 aquatic samples were analyzed, and the OTU composition differed significantly by sampling occasion (PERMANOVA, F = 1.368, R2 = 0.396, P = 0.0001), but not by sampling location (PERMANOVA, F = 1.159, R2 = 0.033, P = 0.073). The result of NMDS ordination also showed temporal variation with the sampling occasion and month (Fig. 1). Subsequent analyses were performed on a total of 24 samples by merging samples from the same sampling occasion. The results of PERMANOVA showed that the OTU composition differed significantly by sampling month, but not by sampling year (Fig. 1 and Table 1; PERMANOVA, month, P = 0.0001, year, P = 0.461). A similar temporal pattern was observed in the results of the GAM regressions (Table 2), where the dissimilarity of OTU composition (SES value of Jaccard index) showed a one-year periodicity (Fig. 2). The local maximum values of community dissimilarity were observed at approximately 180 and 540 days, whereas the local minimum values were at 370 days. In addition, the dissimilarity, although periodic, tended to increase over time (as the x-axis increased). For example, the overall dissimilarity values were higher after approximately 600 days than after approximately 180 days.

Temporal patterns of OTU composition showed a similar trend in individual guilds and the fungal OTU dataset. The OTU composition differed from month to month, regardless of the year in all guilds of fungi (Table 1, PERMANOVA, $P < 0.05$). Furthermore, the dissimilarity of OTU composition in all guilds showed a one-year periodicity (Fig. 2). The strength of periodicity varied across guilds; the explanatory power of sampling months in PERMANOVA, the periodicity of OTU composition dissimilarity (SES value), and the explanatory power of GAM were lower for symbionts than for saprotrophic and parasitic fungi (Tables 1 and 2, Fig. 2). The occurrence patterns of individual OTUs varied. First, there were OTUs that showed seasonal occurrence that were detected in the same month of different years. For example, OTU_0007 (Ramularia sp., plant pathogen) was detected from November to February every year, and OTU_2765 (Filosporella sp., saprotroph) was detected from February to May every year. However, many OTUs did not have a cyclic pattern of occurrence. For example, OTU_0646 (Mycena sp.,

saprotroph) and OTU_1103 (Inocybe sp., symbiont) were detected only in the first year. In addition, 2171 OTUs (49%) were detected in only one sampling occasion over the two years. To provide an overview of the pattern of occurrence of individual OTUs, the typical occurrences of OTUs in each guild are shown in Fig. S5.

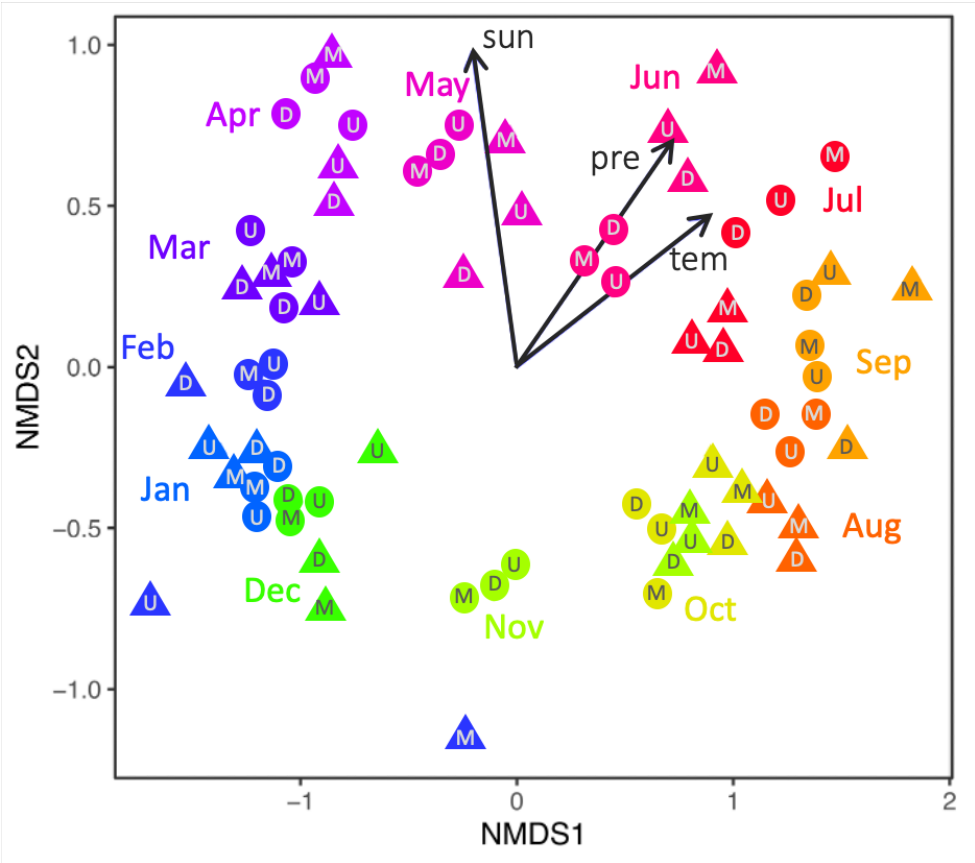

Figure 1. Dissimilarity in the DNA assemblages among sampling occasions, as revealed via nonmetric multidimensional scaling (NMDS) ordination (stress value = 0.1412). Circles indicate the first year of sampling, and triangles indicate the second year of sampling. The colors indicate sampling months. The characters within the points indicate the collection location. The location indicated by each character is shown in Figure S1. Arrows indicate the average air temperature, accumulated precipitation, and total sunshine hours two weeks before each sampling date at the study site (see Table S1).

Table 1. PERMANOVA results for the composition of DNA assemblages

	year			month		
	F value	R^2	P-value	F value	R^2	P-value
all	0.9500	0.04139	0.4685	1.5804	0.59161	0.0001
Fungi	0.9219	0.04022	0.5428	1.5483	0.58665	0.0001
Saprotroph	0.8244	0.03612	0.7711	1.4337	0.56789	0.0001
Parasite	0.9410	0.04102	0.5216	1.4529	0.57115	0.0001
Symbiont	0.7204	0.03171	0.9296	1.2806	0.54000	0.0032

Table 2. GAM results for the relationship between the compositional dissimilarity of OTU composition and temporal distance

	F value	Adjusted R^2	P-value
all	56.93	0.643	< 0.0001
Fungi	40.77	0.557	< 0.0001
Saprotroph	15.96	0.305	< 0.0001
Parasite	25.70	0.433	< 0.0001
Symbiont	4.91	0.103	< 0.0001

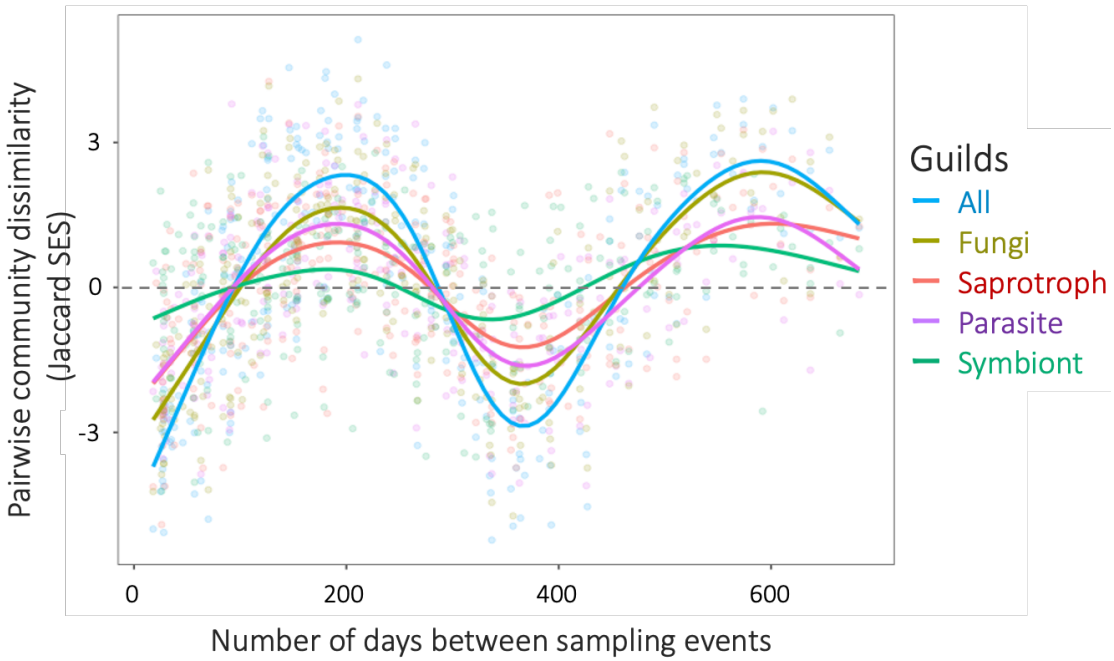

Figure 2. The relationship between the dissimilarity of OTU composition and temporal distance revealed via GAM. The regressions had significant coefficients for all fungi and each functional group (P < 0.0001, see Table 2).

Variation partitioning was used to estimate the relationships between climatic and temporal variables and the temporal changes in OTU composition (all fungal OTUs and individual guilds). For the climate variable, the mean daily air temperature (T2w) was selected as a significant variable for all OTUs and for all guilds. In addition, the solar radiation hour (S2w) was selected for all OTUs and parasitic fungi, and cumulative precipitation (P3d) was selected for the saprotrophic fungi. The relationship between OTU composition and the time was estimated using temporal distance vectors (Moran's eigenvector maps, MEM), which represent various time patterns, including periodicity. For the MEM vectors, MEM3 and MEM4 were selected as significant vectors for all OTUs and for all guilds. These vectors represented an approximately one-year periodicity (Table S1). In addition, MEM8 and MEM2 were selected for all OTUs, MEM2 for parasitic fungi, and MEM11 and MEM2 for saprotrophic fungi. A list of selected variables is presented in Table S3. The climatic and temporal distance variables explained 6.6–16.8% and 6.3–24.7% of the OTU composition, respectively (Fig. 3). Of the fractions explained, 60–99% were shared between climate and temporal distance variables (Fig. 3).

Comparison of the fungal DNA detected in stream water and other substrates

Additional surveys of soil and vegetation to estimate the source of eDNA detected in the stream water were conducted in 2021, and 974 and 394 of the fungal OTUs were detected in soil and vegetation, respectively (Table S2). Of the OTUs detected in water, 596 (13.5%) were detected in soil or plant materials, although they were detected at different times during the survey. Of these, 352 OTUs were shared between water and soil, 184 were shared between water and plants, and 60 were shared between water, soil, and plants (Fig. S6). The percentages of taxonomic and functional groups among the fungal OTUs detected in the soil and plant matter are shown in Figs. S3 and S4.

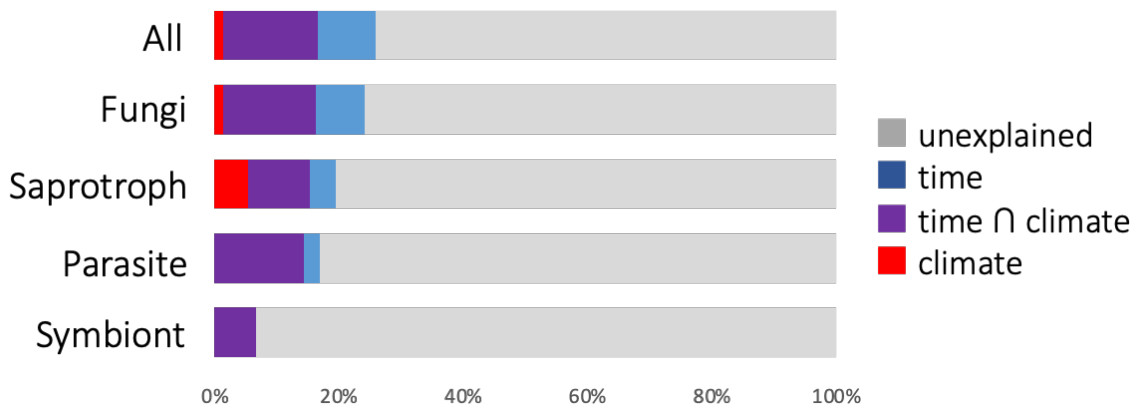

Figure 3. Bar plots showing pure and shared effects of climatic and temporal variables on the fungal OTU assemblages, as derived from variation partitioning analysis. Numbers indicate the proportions of explained variations.

Discussion

In the present study, we reported, for the first time, the temporal patterns of fungal DNA assemblages in a forest stream over two years, by conducting surveys in a flow-regulated forest stream. The fungal DNA assemblage in the stream water contained DNA from both putative aquatic and terrestrial fungi. The DNA assemblages changed over time, and for a given sampling occasion, the differences in the DNA assemblages between sampling locations were fewer than the differences in time (i.e., spatial variation was less than temporal variation (Fig 1). In particular, the DNA assemblages showed a distinct one-year periodicity, meaning that the assemblages were similar to each other regardless of the year when the sampling season was the same (Figs. 1 and 2). These temporal variations were partially explained by climatic variables. Furthermore, the strength of one-year periodicity may vary across functional groups.

Temporal patterns in the fungal DNA assemblages

Temporal patterns in the fungal DNA assemblages showed some agreement with known patterns of temporal dynamics of fungal community composition and/or spore release. First, the relatedness of temporal dynamics of community composition and climate could partly reflect the seasonality of fungal occurrence corresponding to the phenology of host organisms, including plants, plankton, and insects (e.g., Voříšková et al., 2013; Grossart et al., 2019). In the present study, a number of plant saprotrophic and parasitic fungi, both aquatic and terrestrial, were detected. These included OTUs that had seasonal periodicity, such as those detected in the same month of different years. For example, OTU_0007 (Ramularia sp., plant pathogen) was detected from November to February every year and was also detected in plant leaves (Table S2). These fungal DNA can enter the stream along with the host tissue (e.g., tree leaves) (Voronin, 2014). In this case, temporal variations in fungal DNA in the stream may reflect plant phenology, such as seasonal increase in host species abundance and defoliation. These phenologies often correspond to climate seasonality, such as temperature variations (Cleland et al., 2007; Doi and Takahashi, 2008; Kitayama et al., 2021). In fact, tree defoliation increased at the study site from November to February, when air temperature and solar radiation hours decreased. Therefore, fungal DNA with seasonal occurrence patterns, such as OTU_0007, may contribute to the seasonal patterns of the whole DNA assemblage in the stream water by entering the stream along with the host tissue. Second, spore release (i.e., formation of fruiting bodies) by fungi in the surrounding area could also be a source of seasonality. For example, in a forest near the study site (4 km east), formations of fungal fruiting bodies have been reported to show seasonal periodicity, partly explained by seasonal changes in climate, including temperature (Sato et al., 2012). For example, in the area around the study site, the occurrence of fruiting bodies of Boletus species is concentrated from July–September (Sato et al., 2012); in fact, the DNA of several Boletus species has been detected in the stream water between July and September (e.g., OTU_1094 and 1136, Table S2). As more than 90% of spores have been reported to fall within a few kilometers of the fruiting body (Galante et al., 2011; Peay et al., 2012), it is likely that the seasonality of fruiting

body occurrence around the stream via spore recruitment is partly responsible for the temporal variation in the DNA community in water.

The difference in the intensity of seasonal periodicity among functional groups is another correlation between the temporal dynamic patterns observed in the present study and those previously reported (Tables 1 and 2, Fig. 2). Saprotrophic and parasitic fungi showed a relatively distinct periodicity compared to symbiotic fungi (mainly ectomycorrhizal fungi). This may be because a relatively large number of OTUs of saprophytic and parasitic fungi showed a cyclic pattern of occurrence, appearing in the same month in different years, while many OTUs of symbiotic fungi did not have a cyclic pattern, as they occurred only in one year (Table S2). These results may reflect a pattern of seasonal periodicity in the ectomycorrhizal community, which is less cyclic than that of saprotrophic and parasitic fungi. To date, only a limited number of studies have examined the seasonal variations of fungal communities over multiple years; however, some studies in terrestrial ecosystems have reported results consistent with our findings. For example, in fungi associated with plant leaves, seasonal changes in fungi that are consistent with the host plant phenology (e.g., defoliation) have been reported (Osono, 2008; Voříšková et al., 2013). In contrast, in the ectomycorrhizal community, seasonal periodicity may be weak despite time-related community changes (Matsuoka et al., 2016; Sugiyama et al., 2020). Therefore, the temporal variation of fungal DNA assemblages in water may partially reflect the temporal variation of fungi in the peri-stream ecosystem, suggesting that forest streams act as a "natural trap" for fungal DNA, and that eDNA in streams could provide information about the temporal variations in fungal communities in a forest. However, this study is based on simple observations in a flow-regulated stream, and many OTUs were not assigned to taxonomic or functional groups. Therefore, the generality of the observations, the factors that cause temporal changes in the community, and the contribution of individual taxa/functional groups to the temporal change patterns of the community remain open issues. For example, if fungal DNA in the stream water partly reflects the fungal diversity of the surrounding terrestrial area and mainly enters the stream with rainfall, then the temporal pattern of fungal DNA in the stream could be influenced by the area surrounding the study site, the heterogeneity of the forest, and the seasonal climatic patterns, including rainfall and wind. The relationship between the environment of the study site and the temporal pattern of the fungal DNA assemblage is an important topic for future research. In addition, most of the temporal patterns of the DNA assemblages remain unexplained (Fig. 3). This may be partially because many of the fungal DNAs detected did not have distinct seasonality; another factor could be the other environmental variables that affect the DNA assemblage, such as seasonal changes in water quality. Nevertheless, given the paucity of information on temporal variations in fungal communities and the challenges involved in their study, our results highlight the importance and usefulness of eDNA approaches for fungal community studies.

Fungal DNA in water and its dynamics

The fungal DNA detected in the stream water contained both presumed terrestrial and aquatic fungi. This is consistent with previous studies (Deiner et al., 2016; LeBrun et al., 2018; Matsuoka

et al., 2019). The aquatic fungi included chytrid fungi and aquatic hyphomycetes, which include decomposers of plant substrates and parasites of phytoplankton (Grossart et al., 2019). DNA of these fungi derived from spores and mycelial fragments released into the water, may have been detected. In contrast, a number of OTUs that are thought to be terrestrial fungi have been detected. In particular, 596 OTUs detected in the stream water were also detected in the soil and plant tissues from the study site (Table S2, Fig S6). Although this soil and plant tissue sampling is preliminary, the results suggest that DNA of terrestrial fungi present in the soil and plant tissues at the study site may be recruited into the stream. For these terrestrial fungi, spores and/or mycelial fragments released on land may have entered the stream (Voronin, 2014). For example, in fungi that produce fruiting bodies on the ground or on the host surface, released spores can enter the stream either directly or with rainfall. It has been reported that air and raindrops contain a variety of terrestrial saprotrophic, parasitic, and symbiotic fungal DNA (Chen et al., 2018; Castaño et al., 2019; Redondo et al., 2020). In addition, the DNA of hypogeous fungi that do not form notable fruiting bodies above ground, has also been detected (e.g., Tuber and Elaphomyces, known as ectomycorrhizal fungi). In these fungi, spores and mycelial fragments in the soil may have entered the stream from water flow at the soil surface due to rainfall. Some fruiting bodies of fungi whose DNA has been detected, such as Tuber, were found at the study site (Matsuoka, personal observation).

The fungal DNA in stream water and its temporal variation may be related to the fungal communities in and around the stream, in part due to the recruitment of fungal tissue found there (Deiner et al., 2016; Matsuoka et al., 2019). However, source of the fungal DNA detected in water, status of the DNA source (e.g., spores or mycelia), rate of DNA recruitment and movement (settling) in water, and the rate of DNA degradation in water remain to be determined. In addition, fungal spores can travel thousands of kilometers, although in small amounts (Wilkinson et al., 2012). Therefore, it is necessary to compare the spatial and temporal changes in fungal DNA assemblages in stream water with those of substrates other than the surrounding water, and to examine how DNA is recruited into and degraded in the stream. By addressing these points, we can provide a more robust interpretation of the data obtained using eDNA, such as how much of the fungal DNA in water reflects the diversity of fungi, and on which substrates and over which spatial ranges this diversity occurs.

Technical challenges

There are several technical considerations related to the detection of fungal DNA in water. First, there are primer and database issues, as noted by Matsuoka et al. (2019). In the present study, we used the ITS region as a DNA marker, which is a common DNA barcoding marker in fungi (Schoch et al., 2012), but the SSU and LSU regions of rDNA, rather than ITS, are used as DNA barcoding regions in ancestral lineages of fungi, such as chytrids and Glomeromycota (Nilsson et al., 2019). In the present study, approximately 40% of all OTUs could not be identified, which could be partly due to differences in barcoding regions, particularly in the ancestral lineages. In addition, the environmental sequences often returned unknown lineages of fungi, and some of

the OTUs for which taxonomic and functional groups could not be estimated in the present study, may be due in part to insufficient DNA and functional data in the database (Khomich et al., 2018; Grossart et al., 2019). Recently, the long-read sequencing technology (e.g., Oxford Nanopore) has been used to decipher the SSU-ITS-LSU region to infer the phylogenetic position of OTUs, with no closely related sequences registered in the database (e.g., Tedersoo et al., 2020). Such techniques could be useful for further analyses of fungal DNA in water. In addition, in the present study, the number of OTUs belonging to the phylum Glomeromycota was low. Since there may be a bias in the taxa detected due to bias of the primers used in this study, selection of appropriate primers according to the purpose of the research is important. In addition, sampling and DNA extraction methods will be issues for future studies. For example, for bacterial DNA in water, extraction methods have been shown to affect DNA diversity (Ushio, 2019). As there are known examples of how experimental conditions and sequencing platforms affect metabarcoding results in terrestrial fungi (e.g., Lindahl et al., 2013; Song et al., 2015), it is necessary to examine how experimental conditions affect the extraction results of fungal DNA in water.

Conclusion

In the present study, two-year monitoring of eDNA in a flow-regulated stream revealed that fungal DNA assemblages in water exhibited cyclical temporal variation. Differences in OTU composition due to differences in water sampling locations were small when the sampling time was the same. Furthermore, similar to the results of previous studies, the fungal DNA assemblages in water contained fungal DNA presumed to be aquatic and terrestrial. Our results suggest that forest streams act as a "natural trap" for fungal DNA, and that studies on stream water may provide information on the temporal variation of fungal communities living not only in the water but also in the surrounding ecosystem. Despite several technical limitations, this method may enable long-term monitoring of fungal community compositions in forest ecosystems at lower sampling costs; moreover, disturbance to the system is minimal compared to that caused by conventional approaches that target individual substrates. An important future research topic will be the elucidation of dynamic processes, such as the recruitment into, and loss of fungal DNA from, water, as well as the quantification of temporal changes in individual OTUs, and the identification of causal factors (Ushio, 2019; Ushio, 2020).

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Data Accessibility

The sequence data were deposited in the Sequence Read Archive of the DNA Data Bank of Japan (accession number: DRA011182).

Author Contributions

SM and HD designed the study, and SM, YShimono, and YSugiyama conducted field sampling. SM, YSugiyama, and MU contributed to the molecular experiments. SM, YSugiyama HD, and MU analyzed the data and interpreted the results. SM wrote the initial draft of the manuscript. All authors critically reviewed the manuscript.

Appendix 1. The detailed procedure of molecular analyses.

For MiSeq sequencing, the fungal internal transcribed spacer 1 (ITS 1) region of rDNA was amplified. The first-round PCR (first PCR) amplified the ITS1 region using the ITS1-F-KYO2 and ITS2-KYO2 primer set. An Illumina sequencing primer and six random bases (N) were combined to produce each primer. Thus, the forward primer sequence was: 5′- *ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT* NNNNNN TAG AGG AAG TAA AAG TCG TAA -3′ and the reverse primer sequence was: 5′- *GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T* NNNNNN TTY RCT RCG TTC TTC ATC- 3′. The italic and normal letters represent MiSeq sequencing primers and fungi-specific primers, respectively. The six random bases (N) were used to enhance cluster separation on the flowcells during initial base call calibrations on MiSeq. The 1st PCR was performed in a 12 µl volume with the buffer system of KODFX NEO (TOYOBO, Osaka, Japan), which contained 2.0 µl of template DNA, 0.2 µl of KOD FX NEO, 6.0 µl of 2× buffer, 2.4 µl of dNTP, and 0.7 µl each of the two primers (5 µM). The PCR conditions were as follows; an initial incubation for 2 min at 94°C followed by 5 cycles of 10 s at 98°C, 30 s at 68°C for annealing and 30 s at 68°C, 5 cycles of 10 s at 98°C, 30 s at 65°C and 30 s at 68°C; 5 cycles of 10 s at 98°C, 30 s at 62°C and 30 s at 68°C; 25 cycles of 10 s at 98°C, 30 s at 59°C and 30 s at 68°C, and a final extension of 5 min at 68°C. Eight replicate first-PCRs (per sample) were performed to mitigate the reaction-level PCR bias. Then, the duplicated first PCR amplicons (per sample) were combined, resulting in a template per sample for the second PCR. The PCR templates were purified using Agencourt AMPure XP (PCR product: AMPure XP beads = 1:0.8; Beckman Coulter, Brea, California, USA) before the second PCR.

The second PCR amplified the first PCR amplicons using the primers (forward) 5′-*AAT GAT ACG GCG ACC ACC GAG ATC TAC AC* XXXXXXXX TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-3′ and (reverse) 5′-*CAA GCA GAA GAC GGC ATA CGA GAT* XXXXXXXX GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G- 3′. The italic and normal letters represent the MiSeqP5/P7 adapter and sequencing primers, respectively. The 8X bases represent dual-index sequences inserted to identify different samples. The second PCR was carried out with 12 µl reaction volume containing 1.0 µl of template, 6 µl of 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, Washington, USA), 1.4 µl of each primer (2.5 µM), and 2.2 µl of sterilized distilled water. The PCR conditions were as follows; an initial incubation for 3 min at 95°C followed by 12 cycles of 20 s at 98°C, 15 s at 72°C for annealing and extension, and a final extension of 5 min at 72°C.

The indexed second PCR amplicons were pooled to make a library to be sequenced on MiSeq. The volume of each sample added to the library was adjusted to normalize the concentrations of each second PCR product. The pooled library was purified using Agencourt AMPure XP. A target-sized DNA of the purified library (approximately 380–510 base pairs [bp]) was then excised using E-Gel SizeSelect (ThermoFisher Scientific, Waltham, MA, USA). The double-stranded DNA concentration of the library was then adjusted to 4 nmol/L using Milli-Q water, and the DNA sample was applied to the Illumina MiSeq platform at Kyoto University, Japan.