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

Shunsuke Matsuoka[1], Yoriko Sugiyama[2], Yoshito Shimono[3], Masayuki Ushio[4,5], Hideyuki Doi[1]

1. *Graduate School of Simulation Studies, University of Hyogo 7-1-28 Minatojima-minamimachi, Chuo-ku, Kobe, 650-0047, Japan*
2. *Graduate school of human and environmental studies, Kyoto University, Kyoto 606-8501, Japan*
3. *Graduate School of Bioresources, Mie University, 1577 Kurima-machiya, Tsu, Mie 514-8507, Japan*
4. *Hakubi Center, Kyoto University, Yoshida-honmachi, Sakyo-ku, Kyoto 606-8501, Japan*
5. *Center for Ecological Research, Kyoto University, Hirano 2-509-3, Otsu, Shiga 520-2113, Japan*

Corresponding author: Shunsuke Matsuoka (code_matuoka@hotmail.com)

Abstract

Investigation of the seasonal variation in the fungal community is essential for understanding biodiversity and its 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, including both aquatic and terrestrial species, has been explored for its usefulness in biodiversity surveys. Examining eDNA may allow for the survey of the community over time with less disturbance to the ecosystem. In this study, we assessed whether seasonality of fungal communities can be detected with monitoring of eDNA in a flow-regulated stream in a restored forest. We conducted monthly water sampling in the stream over two years, and used DNA metabarcoding to estimate the taxonomic and functional groups of fungal eDNA in the water. The river water contained taxonomically and 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. These seasonal changes were partially explained by temperature alterations. 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 on the temporal variation of fungal communities inhabiting not only water but also the surrounding ecosystem.

Keywords:

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

Introduction

Fungi include various functional groups such as decomposers, symbionts, and parasites, and thus they 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). Because 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 & 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 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 sequencers has enabled the simultaneous processing of a large number of samples parallelly, and information on the spatial variations of terrestrial and aquatic fungi in various ecosystems is rapidly accumulating (Peay et al., 2016; Nilsson et al., 2019; Grossart et al., 2019). In contrast, studies on the temporal variations are far less frequent.

Typical factors causing temporal variation in the community include seasonal fluctuations of climate (e.g., temperature) and host phenology. Although studies on temporal variations in terrestrial and aquatic fungal communities are limited, results showed seasonal changes in community composition and occurrence of fruit bodies (e.g., Sato et al., 2012; Voříšková et al., 2013; Taylor & Cunliffe, 2016). For example, saprotrophic fungi associated with tree leaves show seasonal patterns in response to the supply of tree defoliation as a substrate (e.g., Voříšková et al., 2013). If the occurrence of fungal species and the associated fungal functions change seasonally (e.g., with changes in months and years), 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 their 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), and these surveys are limited, hindering our understanding of fungal community seasonality.

Investigating seasonal variations in fungal diversity based on time-series sampling is challenging, particularly in terrestrial habitats. In particular, unavoidable destructive sampling and a high-level of spatial heterogeneity of fungal community composition make large-scale community surveys extremely difficult. First, fungal communities are usually 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 major way to investigate fungal community structure. Thus, to examine temporal changes in fungal diversity, researchers typically collect samples that are close to the original substrate (that no longer exists), assuming that the

community composition in the substrates that are close to the original substrates should be similar to that of the original community. This strategy is often adopted in monitoring studies, but it causes a second problem in terrestrial fungal studies. Second, previous studies examining variations in spatial diversity have shown that turnover of fungi in soil and plant substrates can occur over a few tens of centimeters or several meters (Bahram et al., 2016). Therefore, the time-series community data obtained in the previous studies, which investigated fungal communities in substrates at slightly different locations than the initial ones, can include not only temporal change information, but also spatial community differences. (e.g., Matsuoka et al., 2016; Sugiyama et al., 2020). These spatial and temporal differences cannot be fundamentally distinguished if we rely only on the spatial sub-sampling approach. Furthermore, 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.

Environmental DNA (eDNA) analysis may be used to overcome these difficulties. In recent years, the usefulness of eDNA surveys in biodiversity research has been explored as it allows for the 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 from river water in a forest landscape. This is perhaps because the river water contains spores and mycelial fragments of fungi from the surrounding terrestrial areas that have joined the river (Voronin, 2014). Furthermore, the fungal DNA assemblages were spatially structured; that is, similar DNA assemblages were found between rivers that were 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 in river water may provide information on the diversity in the catchment area, including both aquatic and terrestrial fungi. Because of these characteristics of eDNA, using these surveys in river and stream water may be potentially useful for addressing the above challenges. First, water sampling does not involve the removal and destruction of terrestrial substrates such as soils; therefore, disturbance of an ecosystem is minor compared with conventional survey methods. Second, spatial heterogeneity is likely to be much lower than in terrestrial substrates because of the high mobility of stream water. Indeed, the usefulness of eDNA in capturing seasonal community dynamics is beginning to be 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 the assemblages exhibit.

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 variation, especially 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,

stream flow and other factors were controlled to be within a certain range. Unlike natural forest ecosystems, there is very little flooding and sediment runoff caused by heavy rainfall; therefore, the effects of the disturbance are minimized in the system, which allows us to focus on seasonal changes. Continuous water sampling was conducted once a month for two years. The fungal assemblages in the water samples were analyzed via DNA metabarcoding. We identified the taxonomic and functional groups by checking against the database and evaluating their 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 is a 30-year-old secondary forest (Fig. S1, approximately 0.6 ha) dominated by deciduous oak trees (*Quercus serrata*) and evergreen trees (*Castanopsis sieboldii*), which are the main natural vegetation of the area. A small stream runs through the forest and drains the groundwater. The stream covers an area of 592 m^2 and the flow is controlled to be approximately 0.5 L per second. The mean annual 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. 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). To each bottle, 1 mL of the ammonium compound benzalkonium chloride 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 the 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 the bead tubes provided with the kit and vortexed for 20 min. They were then extracted according to the protocol provided with the kit.

The mean daily temperature (T), cumulative precipitation (P), and total solar radiation hours (S) for 3 days (3 d) and two weeks (2 w) prior to the survey date were obtained from the AMEDAS. The mean daily temperature for 3 d and 2 w is expressed as T3d and T2w, respectively. These cumulative durations (3 d and 2 w) were chosen 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 run-time costs. The climatic variables at each sampling occasion are shown in Table S1.

Molecular identification of fungi

The procedures used for molecular experiments and bioinformatics followed 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 set (Toju et al., 2012). The PCR amplicons were pooled and sequenced with 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). 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/). The forward and reverse sequences were then merged. A total of 2,169,706 reads (30,135 ± 12,751 reads per sample, mean ± SE, n = 72) 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 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 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 bioinformatics 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 whose reads were less than 0.0002% of 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 contaminations. After this process, we obtained 1,935,576 reads and 4,388 OTUs. The number of sequences, taxa, functional groups, and consensus sequences of the obtained OTUs are 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 the raw data for further analyses without rarefying the data. For each sample, the presence or absence of OTUs was recorded. Binary data were used for all 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 as well as for each functional group (i.e., saprotrophic, symbiotic, and parasitic fungi). To evaluate temporal variations in OTU compositions among sampling occasions, presence/absence data for each sampling occasion were merged. First, 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: $(Dis_{obs}–Dis_{null})/Dis_{sd}$, where Dis_{obs} is the observed dissimilarity, Dis_{null} is the mean of the null distribution of dissimilarity, and Dis_{sd} is the standard deviation of the null distribution. Null distribution was calculated based on 9999 randomizations preserving both the site of occurrence and the OTU richness with 'randomizeMatrix' command in '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 'gam' command in the 'mgcv' package ver. 1.8-31.

Finally, to estimate the contribution of climatic and temporal factors on the temporal changes of OTU composition, variation partitioning based on a distance-based redundancy analysis was conducted using 'capscale' command in the 'vegan' package. The relative weight of each fraction (pure and shared fractions and unexplained fractions) was estimated following the methodology described by Peres-Neto et al. (2006). The Raup-Crick dissimilarity matrix for each sampling occasion was used. The detailed methods for variation partitioning are described in Matsuoka et al. (2016). Briefly, we constructed the 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 with 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 represent various temporal patterns, such as periodicity. We first created a temporal distance matrix between each sampling occasion based on 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 R^2 values (Peres-Neto et al., 2006).

Results

Fungal diversity

A total of 1,935,576 reads from 72 samples were grouped into 4,388 OTUs with 97% sequence similarity (Table S2). The number of OTUs per sample was 350 ± 109 (mean ± SE). In total, 1,680 OTUs were assigned as Ascomycota (38.4% of the total number of fungal OTUs), 732 as Basidiomycota (8.8%), 89 as Chytridiomycota (1.9%), 27 as Mucoromycota, two as Blastocladiomycota, and one OTU was assigned as Entorrhizomycota (Fig. S3). The remaining 1,667 OTUs were not assigned to any phylum. At the order level, 1,172 Ascomycota OTUs belonged to 46 orders, 581 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 (218), Hypocreales (178), Capnodiales (97), Helotiales (95), and Xylariales (80). OTU numbers of the common basidiomycete orders were Agaricales (142), Polyporales (79), Tremellales (54), Corticiales (45), and Russulales (34) (Fig. S3). At the genus level, 616 Ascomycota OTUs were assigned to 562 genera, and 428 Basidiomycota OTUs to 376 genera; the remaining OTUs were not assigned at the genus level. The OTU-rich genera of ascomycetes and basidiomycetes were *Penicillium* (22 OTUs), *Colletotrichum* (16 OTUs), and *Fusarium* (13 OTUs), and *Cortinarius* (15 OTUs), *Trechispora* (15 OTUs), and *Tomentella* (14 OTUs), respectively. The detailed taxonomic assignments are shown in Table S2.

FUNGuild assigned 1,417 OTUs to functional guilds, 695 OTUs of which were saprotrophs, and the others included parasites (219 OTUs) and symbionts (128 OTUs) (Table S2, Fig. S4). The remaining 375 OTUs included endophytes and OTUs with multiple functions. The major saprotrophs were plant saprotrophs (190 OTUs), including the two 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 (183 OTUs), such as *Taphrina* and *Ciboria*. Among the symbionts, the major fungi were ectomycorrhizal fungi (103 OTUs), such as *Cortinarius* and *Russula*. These plant parasites and ectomycorrhizal fungi are terrestrial fungi, validating our expectation that terrestrial eDNA can be detected in stream water.

Temporal patterns of fungal OTU compositions and related variables

A total of 72 samples were analyzed and the OTU composition differed significantly by sampling occasion (PERMANOVA, $F = 1.368$, $R^2 = 0.396$, $P = 0.0001$) but not by sampling location (PERMANOVA, $F = 1.159$, $R^2 = 0.033$, $P = 0.073$). The result of NMDS ordination also showed temporal variation with the sampling occasion and month (Fig. S3). 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 found 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 found at approximately 180 and 540 d, whereas the local minimum values were at 370 d. In addition, the dissimilarity, although periodic, tended to increase overall over time (as the x-axis increases). For example, the overall dissimilarity values were higher after approximately 600 d than after approximately 180 d.

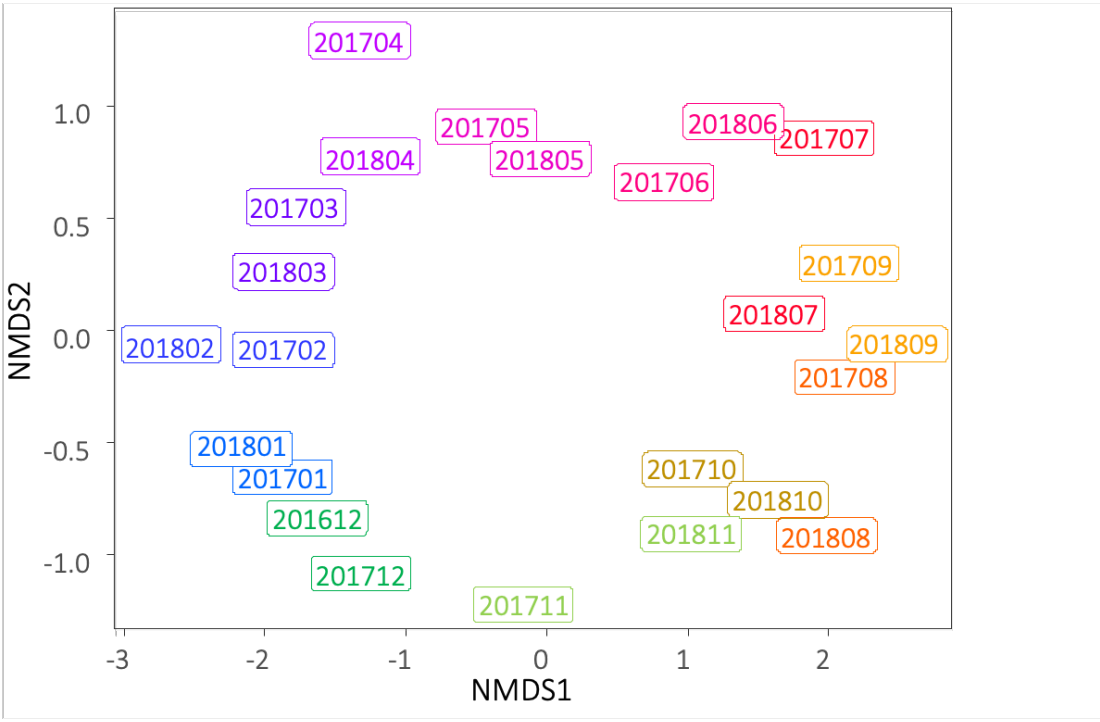

Figure 1. Dissimilarity of the DNA assemblages among sampling occasions as revealed via nonmetric multidimensional scaling (NMDS) ordination (n = 24, stress value = 0.1031). Numbers indicate sampling year and month and are identical with site numbers in Table S1. For example, [201612] means the sample was collected December 2016.

Table 1. PERMANOVA results for the composition of DNA assemblages

	year			month		
	F	R^2	p-value	F	R^2	p-value
all	0.9552	0.04161	0.4713	1.5827	0.59197	0.0001
Saprotroph	0.7608	0.03342	0.8695	1.4012	0.56225	0.0004
Parasite	0.9574	0.04170	0.4770	1.5458	0.58626	0.0001
Symbiont	0.8182	0.03586	0.7938	1.2778	0.53944	0.0047

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

	F	Adjusted R^2	p-value
all	56.16	0.640	< 0.0001
Saprotroph	12.27	0.242	< 0.0001
Parasite	32.47	0.496	< 0.0001
Symbiont	4.96	0.102	< 0.0001

Temporal patterns of OTU composition showed a similar trend in individual guilds. 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 the 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 (Table 1 and 2, Fig. 2).

Variation partitioning was used to estimate the relationships between climatic and temporal variables and the temporal changes in OTU composition (all OTUs and individual guilds). For the climate variable, T2w was selected as the significant variable for all OTUs and for all guilds. In addition, S2w was selected for all OTUs and parasitic fungi, and P3d was selected for the saprotrophic fungi. The relationship between OTU composition and time was based on a temporal distance vector (MEM), which represents 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 approximate 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. The list of selected variables is shown 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 fractions of the climate and temporal distance variables (Fig. 3).

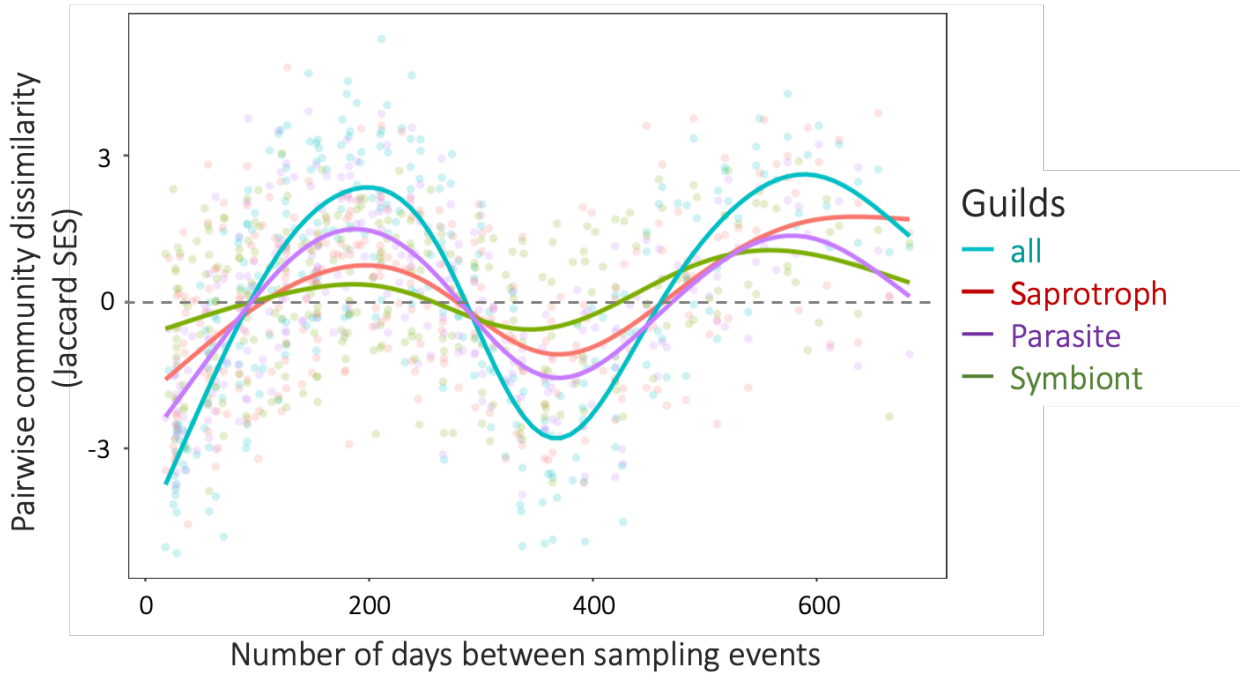

Figure 2. The relationship between the dissimilarity of OTU composition and temporal distance revealed via GAM. The regressions had significant coefficients for all fungi and each functional group ($P < 0.0001$, see Table 2).

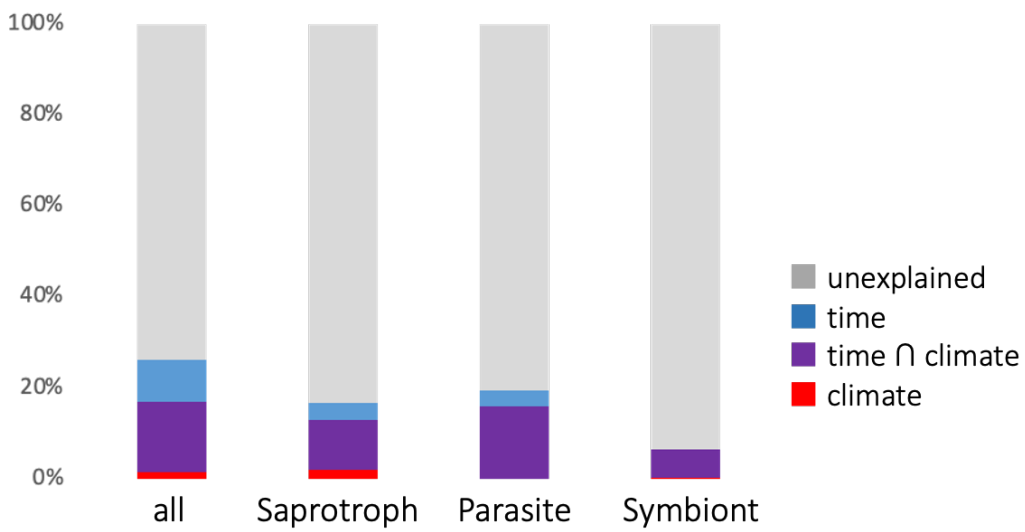

Figure 3. Bar plots showing pure and shared effects of climatic and temporal variables on the fungal OTU assemblages as derived from variation partitioning analysis. Numbers indicate the proportions of explained variation.

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, and the DNA assemblages changed over time. In particular, the DNA assemblages showed a distinct one-year periodicity, meaning that the assemblages were similar to each other regardless of the year, if the sampling season was the same. These temporal variations were partially explained by climatic variables. Furthermore, the strength of the one-year periodicity may vary across functional groups.

Temporal patterns in the fungal DNA assemblages

Temporal patterns in the fungal DNA assemblages show some agreements with known patterns of temporal dynamics of fungal community composition and/or spore release. First, the relatedness of temporal dynamics of community compositions and climates 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, for example, a number of plant saprotrophic and parasitic fungi, both aquatic and terrestrial, were detected. These fungal DNA could 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 increases in host species abundance and defoliation. These phenologies often correspond to climate seasonality, such as temperature variations (Cleland et al., 2007; Doi & Takahashi, 2008; Kitayama et al., 2020). Second, spore release (i.e., formation of fruit bodies) of 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 fruit bodies were reported to show a seasonal periodicity, partly explained by seasonal changes in climate, including temperature (Sato et al., 2012). As more than 90% of spores have been reported to fall within 1 km 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 dynamics patterns observed in the present study and those previously reported (Table 1 and 2, Fig 2). Saprotrophic and parasitic fungi showed a relatively distinct periodicity compared to symbiotic fungi (mainly ectomycorrhizal fungi). These results may reflect a pattern of seasonal periodicity in the ectomycorrhizal community that is less cyclic than that of the saprotrophic and parasitic fungi. To date, only a limited number of studies have examined the seasonal variation of fungal communities over multiple years, but some studies in terrestrial ecosystems have shown 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, the seasonal periodicity may be weak, despite the 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 on the temporal variation of 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. In addition, some temporal patterns may have been omitted due to the unidentified OTUs. Nevertheless, given the paucity of information on temporal variations in fungal communities and the challenges of 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 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. On the other hand, a number of OTUs that are thought to be terrestrial fungi have been detected. 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 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 DNAs (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 the water flow at the soil surface, due to rainfall. Some fruit bodies of fungi whose DNA has been detected, for example *Tuber*, were found at the study site (Matsuoka personal observation).

The fungal DNA in stream water and its temporal variation may relate 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, the source of the fungal DNA detected in water, the status of the DNA source (e.g., spores or mycelium), the 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 potentially travel thousands of kilometers, although in small proportions (Wilkinson et al., 2012). Therefore, it is necessary to compare the spatial and temporal changes of the fungal DNA assemblages in stream water with that of the substrate other than the surrounding water, and to examine how DNA is recruited and degraded in the stream.

By addressing these points, we can provide a more robust interpretation of the data obtained by eDNA, such as how much of the fungal DNA in water reflects the diversity of fungi, and on which substrates and spatial ranges this diversity occurs.

Technical challenges

There are several technical considerations in relation 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 (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, especially in the ancestral lineages. In addition, the environmental sequences often return 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 (Grossart et al., 2019; Khomich et al., 2018). Recently, 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). These techniques will be useful for further analyzing fungal DNA in water. In addition, sampling and DNA extraction methods are issues for future studies. For example, for bacterial DNA in water, extraction methods have been shown to affect the diversity of DNA detected (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 fitting to examine how experimental conditions affect the results of fungal DNA in water.

Conclusion

In the present study, a two-year monitoring of eDNA in a flow-regulated stream revealed that fungal DNA assemblages in water exhibited a 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 community 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 the surrounding ecosystem. Despite several technical limitations, it may enable long-term monitoring of fungal community compositions in forest ecosystems at lower sampling costs, and disturbance to the system is minimal compared to conventional approaches that target individual substrates. An important future research topic will be the elucidation of dynamic processes such as the recruitment and loss of fungal DNA in 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.