The occurrence patterns of gut bacteria in a post-mined peatland, northern Japan

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SUMMARY

The spatio-temporal fluctuations of gut bacteria were monitored in peat-pore water collected from post-mined peatlands in Sarobetsu Mire, northern Japan, where various mammalian species are present, during snow-free periods from spring 2018 to autumn 2019. The composition of gut bacteria was measured by operational taxonomic units (OTUs) in four successional habitats: bare ground, sedgeland, grassland and moss (*Sphagnum*) mat. Of the 140 peat-pore water samples collected, 129 samples contained gut bacteria. The total of 56 gut-bacteria OTUs found comprised 5.10 % of all the 1,097 taxa, while the gut bacteria accounted for a mean 0.07 % and maximum 1.71 % of relative dominance. The relative dominance of gut bacteria fluctuated seasonally at various taxonomic levels. Although the total read numbers of bacteria did not differ between the four habitats, the read numbers of gut bacteria in Clostridiales, Bacterioidiales and Lactobacteriales tended to show higher dominance in vegetated habitats. These results lead us to hypothesise that vegetation development is related to the richness and abundance of gut bacteria.

KEY WORDS: gut bacterial communities, vertebrate scats, 16S rRNA gene amplicon sequencing

INTRODUCTION

The structure of the microbiota community is important in determining ecosystem structure and function in wetlands (Bahram *et al.* 2022). Understanding the immigration source of bacteria is one of the keys to understanding environmental microbiota in post-disturbance conditions because the immigration sources determine the succession of taxon composition (Jackrel *et al.* 2019). Mammals contribute such bacterial sources through their faeces (Wang *et al.* 2022). Large mammals observed in the wetlands of northern Japan - such as deer, fox and hare - exhibit seasonality in behaviours because of seasonal changes in the availabilities of food resources, breeding sites, etc. (Abe & Ota 1987). In addition, the environmental microbiota is often affected by the rhizosphere development that is associated with above-ground vegetation structure (Tkacz *et al.* 2020). These observations indicate that the occurrence of gut bacteria in the external environment may change rapidly and/or seasonally across succession gradients (Cobaugh *et al.* 2015). Similarly, if the gut bacteria supplied from faeces are short-lived in the external environment, the densities of environmental microbiota emanating from gut bacteria may exhibit high temporal fluctuations (Douglas 2018).

After catastrophic disturbances, including large-scale soil removal, primary succession starts with bare ground where no rhizosphere develops, with bacteria thus developing colonies without a rhizosphere present (Ciccazzo *et al.* 2016). In the early stages of succession after large disturbances, such as peat mining or volcanic eruptions, above-ground phytomass increases with below-ground phytomass (Hirata & Tsuyuzaki 2016), affecting rhizosphere functions including microbial activities (Otaki *et al.* 2016). Vertebrate populations subsequently increase with increasing phytomass and food resources across the succession gradient (Schrama *et al.* 2012). Both the above-ground and below-ground succession processes suggest that the environmental microbiota is affected by the activities of vertebrates such as mammals (Bardgett & Wardle 2003). However, the specific taxa of gut bacteria do not persist long term in peat because of unsuitable habitats (Camiade *et al.* 2020).

Considering this, the seasonal fluctuations of gut bacteria were monitored in post-mined peatland. Two hypotheses were examined: (1) the dominance of gut bacteria in peat pore water will differ among the vegetation types representing a successional sequence, and (2) the dominance and its fluctuations will be higher in areas with denser vegetation. To investigate these hypotheses, a post-mined peatland

in Sarobetsu Mire, northern Japan, was used because the aboveground successional patterns are well known (Nishimura & Tsuyuzaki 2014).

METHODS

The study sites were post-mined peatlands in Sarobetsu Mire, northern Japan (45° 06′ N, 141° 42′ E, 7 m a.s.l.). At the Toyotomi Meteorological Observatory, 6 km from the study site, the 1981–2020 mean annual temperature was 6.1 ºC with a monthly maximum of 23.7 ºC in August and a monthly minimum of -6.5 ºC in January. The 1981–2020 mean annual precipitation was 1072.5 mm. Peat mining was conducted annually, on areas of 3–22 ha and to depths up to 3–6 m, from 1970 to 2003. The two research sites were located in areas mined for peat in 1972 and 1982. Succession after peat mining in these sites largely follows the sequence of four vegetation types (habitats) ordered chronologically by Nishimura & Tsuyuzaki (2014): bare ground, *Rhynchospora alba* (L.) Vahl sedge with low plant cover, *Moliniopsis japonica* (Hack.) Hayata grass with high plant cover and *Sphagnum* (peat moss) mat. The major peat moss species locally is *Sphagnum papillosum* Lindb. (Nishimura & Tsuyuzaki 2014).

Within each of the four habitats mined in 1972 or 1982, three 50 × 50 cm sampling plots were delineated using four plastic sticks installed at the four plot corners in late April 2018 (total of 24 plots). Peat-pore water and peat were collected from these plots in six sampling events between June 2018 and October 2019. Peat-pore water was collected from *Sphagnum* mat in 2018 using a porous cup (Mizu Thoru DIK-8392, Daiki Rika Kogyo Co., Ltd., Saitama, Japan). Peat was collected using a spatula, from *Sphagnum* mat in 2019 and from the other three habitats in 2018 and 2019. The peat and peat-pore water were both collected from 5–10 cm depth. In vegetated plots this depth range overlapped roughly with that of the upper rhizosphere, including the moss layer where *Sphagnum* had established, although *R. alba* and *M. japonica* roots develop at different peat depths (Egawa & Tsuyuzaki 2011). Since the ground surface of post-mined peatland is covered with the residues of peat after mining, the sampling layer in plots without *Sphagnum* consisted of peat only. To study potential seasonality the sampling was conducted three times during the snow-free period of each year, in late spring (late May or mid-June), mid-summer (August) and late autumn (late October or early November). In total, 140 samples were used for analysis, because four samples from *Sphagnum* mat were not measured owing to a paucity of sampled water.

The environmental DNA within the samples was purified using a Water RNA/DNA Purification Kit (Norgen Biotek Corp., Thorold, Canada) and ISOIL (Nippon Gene Co., Ltd., Tokyo, Japan) following the manufacturers' instructions. The purified DNA was subjected to agarose gel electrophoresis to examine the purity and was quantified by measuring absorbance at 260 nm. The V3-V4 variable regions of 16S rRNA genes were analysed following the Illumina protocol for 16S Metagenomic Sequencing Library Preparation (Illumina 2013). Bar-coded amplicons, amplified with 341F and 806R primers, were:

Forward, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′

and Reverse, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′,

and were sequenced using the Illumina MiSeq 2 × 300 bp platform.

The PCR program used for the 16S amplicon was: initial denaturation at 95 ℃ for 3 min, then 25 cycles consisting of denaturation at 95 ℃ for 30 sec, annealing at 55 ℃ for 30 sec and extension at 72 ℃ for 30 sec, followed by the final extension at 72 ℃ for 5 min and kept at 4 ℃ (Illumina 2013). The PCR products of the 16S V3-V4 region were purified with AMPure XP beads (Illumina 2013). One fifth of the PCR products were used as templates in the subsequent barcoding PCR using primer pairs with unique barcodes. Barcoded PCR amplicons were pooled in an equimolar fashion after purification by AMPure XP beads. The sequencing was performed with an Illumina MiSeq sequencing instrument with 300 bp pair end sequencing by the sequencing company FASMAC (Kanagawa, Japan). The sequencing data were analysed with the standard QIIME pipeline and low-quality reads, and adaptor contaminations were eliminated by the sequencing company.

To nominate obligate gut bacteria, we applied two-step screening. First, the taxa of operational taxonomic units (OTUs) in Phylum Fusobacteria, Class Bacteroidia, Orders Enterobacteriales, Bifidobacteriales, Lactobacillales and Clostridiales were listed as potential gut bacteria, based on relevant references (Thomas *et al.* 2011, Guan *et al.* 2017, Rinninella *et al.* 2019). Secondly, the taxa for which reproduction is not dependent on a vertebrate body (according to the relevant literature) were excluded from the list, and *vice versa* (Table 1).

Table 1. Classified list of 56 gut-bacterium taxa detected in soil and water samples collected from Sarobetsu mire, northern Japan during the snow-free periods of 2018 and 2019. References used to support exclusion or inclusion as gut bacterial taxa are provided (in parentheses).

Phylum	Class	Order	Family	Taxa detected
Bacteroidetes	Bacteroidia	Bacteroidales (Yap *et al*. 2014, Tourlousse *et al*. 2015)	Bacteroidaceae	*Bacteroides uniformis* (Robinson *et al*. 1981), *Bacteroides* sp., 5-7N15 sp., genus unassigned
			Porphyromonadaceae	*Candidatus_Azobacteroides* sp. (Pramono *et al*. 2017), *Dysgonomonas* sp. (Bilen *et al*. 2019), *Paludibacter* sp., *Porphyromonas endodontalis*, *Porphyromonas* sp., *Parabacteroides* sp., genus unassigned
			Prevotellaceae	*Prevotella melaninogenica*, *Prevotella* sp.
			Rikenellaceae	Blvii28 sp., PW3 sp., genus unassigned
			[Paraprevotellaceae]	[*Prevotella*] sp., CF231 sp., YRC22 sp.
Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae (Olsten 2014)	*Cetobacterium somerae* (Finegold *et al*. 2003), *Fusobacterium* sp., u114 sp.
			Leptotrichiaceae	*Leptotrichia* sp., *Leptotrichia* sp.
			unassigned	
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae (Cooney *et al*. 2014, Liu *et al*. 2017)	*Klebsiella* sp., *Proteus* sp., *Providencia* sp., *Salmonella* sp. (EFSA 2011)
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium longum* (Quigley 2017)
Firmicutes	Bacilli	Lactobacillales (Lappan *et al*. 2020, Yang *et al*. 2021)	Lactobacillaceae	*Lactobacillus* sp.
			Streptococcaceae (McAuliffe 2018)	*Streptococcus* sp.
			Enterococcaceae	*Enterococcus* sp. (Byappanahalli 2012)
			Leuconostocaceae	genus unassigned
	Clostridia (Clayton *et al*. 2018)	Clostridiales (Kallistova *et al*. 2014, Hatmaker *et al*. 2019, Hur *et al*. 2019)	Christensenellaceae (Waters & Ley 2019)	genus unassigned
			Dehalobacteriaceae	*Dehalobacterium* sp.
			Lachnospiraceae (Bui *et al*. 2014)	*Anaerostipes* sp., *Blautia* sp., *Butyrivibrio* sp., *Coprococcus* sp., *Dorea* sp., *Lachnospira* sp. (Cornick 1994), [*Ruminococcus*] *gnavus*, [*Ruminococcus*] sp., genus unassigned
			Ruminococcaceae (Bell *et al*. 2019)	*Ruminococcus* sp., *Oscillospira* sp.
			Veillonellaceae (Watanabe *et al*. 2021, Shigeno *et al*. 2019)	*Dialister* sp. (Chiu *et al*. 2014, Afouda *et al*. 2020, Sakamoto *et al*. 2020), *Sporomusa* sp., *Phascolarctobacterium* sp., *Veillonella dispar* (Wei *et al*. 2020), genus unassigned (Marhandin & Jumas-Bilak 2014)
			[Acidaminobacteraceae]	*Acidaminobacter* sp. (Weir *et al*. 2013)
			[Tissierellaceae]	*Anaerococcus* sp. (Chen *et al*. 2012), *Peptoniphilus* sp. (Demehri *et al*. 2016), *Finegoldia* sp.

Since the read number estimates the relative expression levels of genes in a cell at a given time, it expresses the abundance of each bacterial taxon (Jian *et al*. 2020). The relative dominance of gut bacteria (%) was calculated as: (sum of gut-bacterium read number) ÷ (total read number) × 100. The relative dominance of each taxon was compared between the vegetation types, sampling dates and mined years using a generalised linear mixed-effects model (GLMM) with an assumption of binomial distribution of dominance and plot codes as a random effect. To extract samples showing high relative dominance of gut bacteria in the peat, a dominance-rank curve was drawn. Box-and-whisker plots were drawn to show outliers that exceeded the first quartile + 1.5 × interquartile range. Tietjen-Moore tests were conducted to re-confirm the outliers that showed unusual dominance at the significance level of 0.01 for conservative estimation. The differences in the read numbers of major gut-bacterial taxa were examined by GLMMs with an assumption of Poisson distribution of read numbers on each of three major OTUs of gut bacteria. All the statistical analyses were performed using R software (version 4.0.0) (R Core Team 2021) with the libraries MASS (Venables & Ripley 2002), lme4 (Bates *et al*. 2015) and EnvStats (Millard 2013).

RESULTS

The total number of OTUs obtained from the 140 samples was 1,097 and the total read numbers ranged from 816 to 393,929 (mean 103,149 ± 66,441 SD). The number of gut-bacterium OTUs was 56 (Table 1), comprising 5.10 % of all the taxa. The gut bacteria belonged to six taxonomic orders (Bacteroidales, Bifidobacteriales, Clostridiales, Enterobacteriales, Fusobacteriales and Lactobacillales) from five phyla: Actinobacteria (Bifidobacteriales), Bacteroidetes (Bacteroidales), Firmicutes (Lactobacillales and Clostridiales), Fusobacteria (Fusobacteriales) and Proteobacteria (Enterobacteriales). Of the six orders, Fusobacteriales and Bifidobacteriales contained below 37 and 5 of the read numbers, respectively, and comprised less than 0.002 % of the total reads. The read number of gut bacteria was below 2,609 in all samples, and eleven (8 %) of the peat samples did not contain any gut bacteria. The read numbers of gut bacteria averaged 81 ± 231 SD per sample with a relative abundance of 0.07 % ± 0.15 SD.

Mammalian faeces were observed to be scattered in the post-mined peatlands, particularly on well-vegetated areas (Figure 1). Based on the morphological traits of scats and pellets observed in

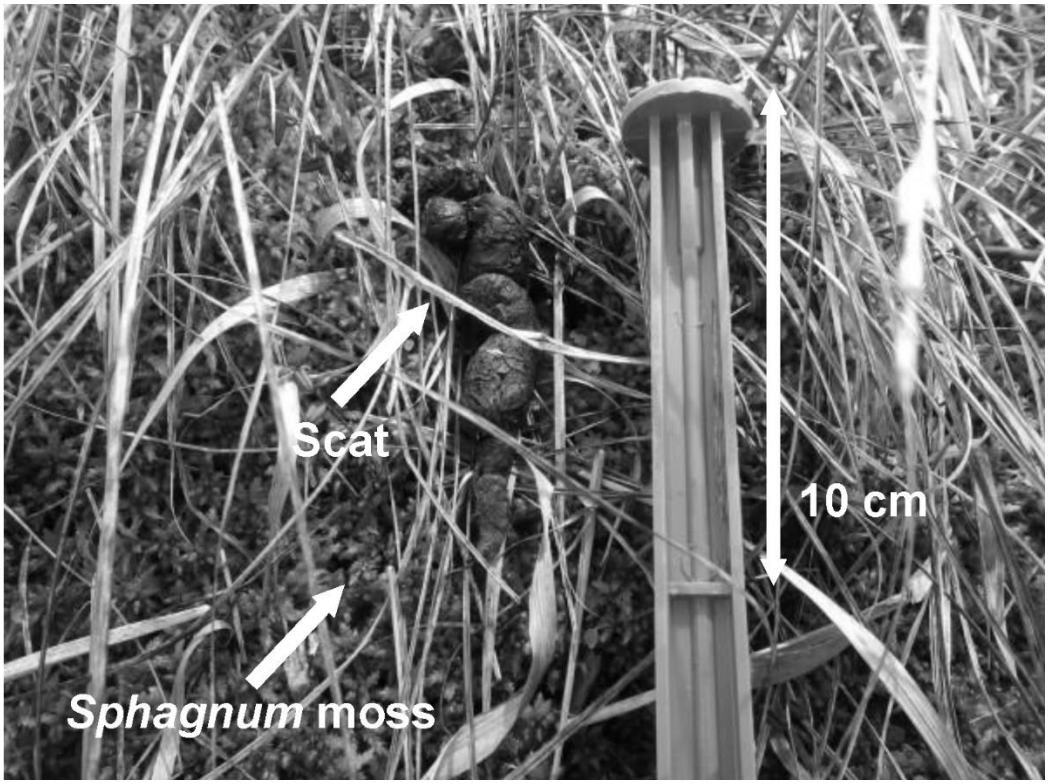

Figure 1. A fresh fox scat spread on a *Sphagnum* moss mat in post-mined peatland at Sarobetsu Mire, northern Japan, on 22 Apr 2020. The site was mined in 1972.

the field, the major providers of mammalian faeces were considered to be: *Cervus nippon yesoensis* Heude, 1884 (Hokkaido sika deer), *Vulpes vulpes schrencki* Kishida, 1924 (Ezo red fox) and *Lepus timidus ainu* Barrett-Hamilton, 1900 (mountain hare).

The dominance-rank curve of gut bacteria showed a high dominance of gut bacteria in four to six samples (Figure 2). From the box-and-whisker plots, six samples were considered to be outliers, whereas the Tietjen-Moore test confirmed that four of the peat samples showed extraordinary dominance of gut bacteria, ranging from 0.308 % to 1.795 % (Table 2). In contrast, 11 samples contained no gut bacteria. These were recorded from bare ground (2 samples), *M. japonica* grassland (1) and *Sphagnum* mat (8) (Figure 2), showing that the persistence of gut bacteria was short or limited in *Sphagnum* mat.

The four outlier samples of gut bacteria were censused in detail, by using the numbers of reads (Table 2). Even within the four outliers, the environmental characteristics were diverse (i.e., mined years and sampling dates were heterogenous). However, three of these four samples were collected from the *M. japonica* grassland and one was from *Sphagnum* mat. Villonellaceae (Clostridiales), consisting of four OTUs (Table 1), showed high dominance in the four peat samples (Table 2). In the largest outlier sample, Porphyromonadaceae dominated by *Paludibacter*, and Christensenellaceae, also showed high numbers of reads. *Candidatus-Azobacteroides* (Porphyromonadaceae) showed the highest read number in the second largest outlier. *Paludibacter* (Porphyromonadaceae) was also extracted from these four samples with high read numbers.

The relative dominance of gut bacteria fluctuated over time in all four habitats (GLMMs, $P < 0.001$), particularly in the *M. japonica* grassland due to Veillonellaceae (Figure 3). GLMM analyses

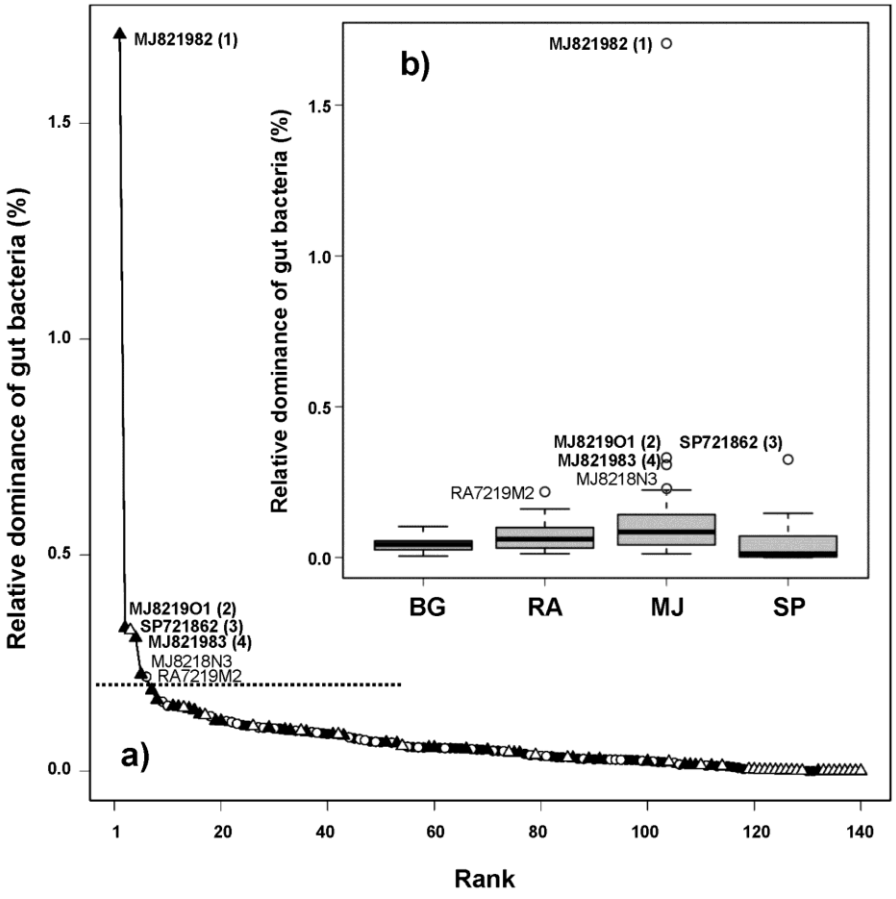

Figure 2. (a) Dominance-rank curve showing the relationship between sample rank from high (rank = 1) to low (140) and the relative dominance of gut bacteria. Closed circles, open circles, closed triangles and open triangles distinguish the vegetation/habitat types bareground (BG), *Rhynchospora alba* sedgeland (RA), *Moliniopsis japonica* grassland (MJ) and *Sphagnum* mat (SP). (b) box-and-whisker plots with outliers shown by circles ($n = 6$) in the four vegetation types. Outliers are labelled with their sample codes, in **bold** if re-confirmed by Tietjen-Moore test. The detailed bacterial compositions of the first four samples (coded as 1 to 4) are explained in Table 2.

Table 2. Numbers of reads on the OTUs of gut bacteria in the four samples that show relatively high occurrence of gut bacteria, coded as 1 to 4. All of these samples are peat. The sample codes correspond to the **bold** outlier labels in Figure 2b.

				Sample code	MJ821982 (1)	MJ8219O1 (2)	SP721862 (3)	MJ821983 (4)
				Vegetation type	*Moliniopsis* grassland	*Moliniopsis* grassland	*Sphagnum* mat	*Moliniopsis* grassland
				Mined year	1982	1982	1972	1982
				Sampling date	21 Aug 2019	24 Oct 2019	22 Jun 2018	21 Aug 2019
				Total read number	152987	192092	65128	154170
				Relative dominance of gut bacteria (%)	1.795	0.332	0.326	0.308
Gut-bacterium Order, Family and taxon	Bacteroidales	Porphyromonadaceae	*Candidatus_Azobacteroides*		26	311	0	5
			Dysgonomonas		20	1	0	75
			Paludibacter		76	4	48	108
			Parabacteroides		0	14	0	46
		Rikenellaceae	PW3		22	11	0	42
			genus unassigned		19	1	1	9
	Fusobacteriales	Fusobacteriaceae	u114		33	37	0	0
	Enterobacteriales	Enterobacteriaceae	*Klebsiella*		15	0	0	4
	Lactobacillales	Streptococcaceae	*Streptococcus*		0	30	9	12
	Clostridiales	Christensenellaceae	genus unassigned		54	22	6	1
		Lachnospiraceae	genus unassigned		10	0	0	1
		Ruminococcaceae	*Ruminococcus*, *Oscillospira*		0	2	0	0
		Veillonellaceae	genus unassigned		2332	205	147	172
			Sporomusa		0	0	1	0
Other gut bacteria			genus unassigned		2	0	0	0

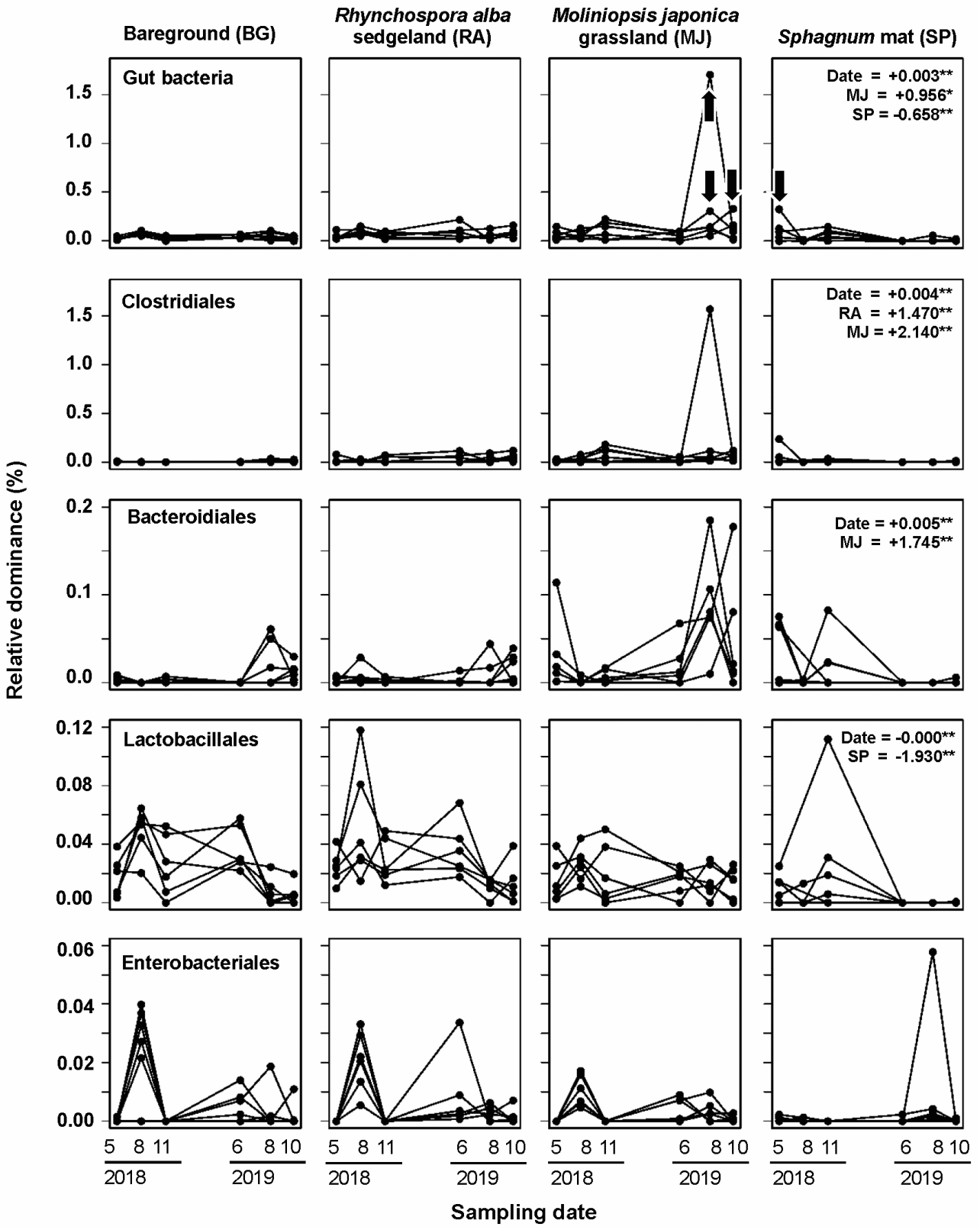

Figure 3. Seasonal fluctuations of gut-bacterium taxa estimated by OTUs in the four vegetation types during 2018 and 2019. The lines connect successive samples from the respective sampling plots. Differences in numbers of reads are examined among sampling dates and between the bareground and other three habitats by GLMM (**: $p < 0.01$, *: $p < 0.05$). The four samples that showed relatively high dominance of gut bacteria are indicated by arrows (see also Table 2). Note that the *y* axis scales differ between rows of panels.

indicated that the numbers of bacteria did not differ between the four habitats ($p > 0.26$), while the read numbers of the total gut bacteria did not differ between the bareground and *R. alba* sedgeland habitats and increased from bareground to *M. japonica* grassland and *Sphagnum* mat ($p < 0.01$). The read numbers differed between sampling dates for the three major taxonomic orders Bacteroidiales, Clostridiales and Lactobacillales ($p < 0.01$), but not for Enterobacteriales ($p = 0.14$), probably because of the small numbers of reads. Bacteroidiales and Clostridiales showed higher read numbers in *M. japonica* grassland than in bareground habitats, and Lactobacillales read numbers were highest in *Sphagnum* mat ($p < 0.01$). Clostridiales also showed higher numbers in *R. alba* sedgeland. Therefore, the habitats where the gut bacteria showed the highest relative dominance differed among the taxonomical orders. The mined year, 1972 or 1982, did not affect the read numbers of total gut bacteria ($p = 0.359$) or any of the orders.

DISCUSSION

Spatiotemporal fluctuations of gut-bacterium microbiota in peat were detected in this study, as well as in water collected from peat-bog lakes in Poland (Lew *et al.* 2019). The gut bacteria exhibited high relative dominance, shown by the outlier analyses, in four to six peat samples, although the dominance and abundance decreased sharply soon after the peaks across the vegetated habitats. Because the high relative dominance of gut bacteria occurred several times but only in vegetated habitats, the abrupt appearance of gut bacteria was unlikely to be random noise. The high fluctuations of relative dominance suggest that the ecological processes were altered by the gut bacteria when the vegetation developed. The gut bacteria generally showed small read numbers but explained more than 1/20 of OTUs, inferring that they played some ecological role in succession after peat mining.

Faeces of deer, fox and hare were visually observed in the field. Faeces play an important role in determining the abundance and spatial distribution of nutrients within an ecosystem, which in turn influences the abundance and distribution of plants and microbiota (Keddy 2017). For example, hare pellets have been found to contribute to seed dispersal on a nutrient-poor volcano (Nomura & Tsuyuzaki 2015, Tsuyuzaki 2020). Small mammals such as rodents and shrews are also found in Sarobetsu Mire (Abe 1984), along with birds whose faeces play a role in seed dispersal (Nishi & Tsuyuzaki 2004). Sarobetsu Mire has been registered as a Ramsar Convention site since 2005 to protect migrant birds (KSNRPC 2007). These authors suggested that diverse gut bacteria were supplied frequently by various vertebrate vectors, although their persistence was not guaranteed. In addition, the microbiota of gut bacteria in vertebrates are highly diverse between and even within species, depending on their environments and diets (Finlayson-Trick *et al.* 2017, Menke *et al.* 2019).

In our study, gut bacteria were more abundant in vegetated habitats (i.e., the *R. alba* sedgeland, *M. japonica* grassland and *Sphagnum* mat) than in those with bare ground, indicating that the occurrence of gut bacteria was enhanced by plant cover and roots. Furthermore, the habitats where the gut bacteria showed high occurrence tended to differ among the taxonomic orders. Of the six outlier samples, four were recorded form *M. japonica* grassland (MJ). *R. alba* and *M. japonica* utilise different peat depths by their distinct root development patterns (Egawa & Tsuyuzaki 2011), suggesting that the gut bacteria composition is affected by rhizosphere structures and further studies are required to confirm this.

At the phylum level, Bacteroidetes (Bacterioidales) and Firmicultes (Lactobacillales and Clostridiales) were often detected in our peat samples. Fusobacteria and Proteobacteria were least commonly found and Tenericutes was not detected. Firmicutes, Bacteroidetes and Tenericutes are predominant phyla in the faeces of wild sika deer in northeastern China (Guan *et al.* 2017). Bacteroidetes and Firmicutes are major taxa in human gut microbiota (Johnson *et al.* 2017). Gut bacteria in ambulatory mammals are highly host-specific, as compared with birds and bats (Song *et al.* 2020). These suggest that the supply of gut bacteria occurs frequently and/or biasedly on the post-mined peatlands 38–48 years after peat mining.

Based on these results, two factors are considered to determine the occurrence of gut bacteria in our samples. First, the behaviour of mammals determines the distribution of gut bacteria in wetlands, because most vertebrates have habitat preferences (Tsuyuzaki & Takahashi 2007, Kawaguchi & Desrochers 2018). For example, mountain hare infrequently utilise grassy habitats, including Sarobetsu Mire, during the snow season to avoid predators (Abe & Ota 1987). Secondly, while the bacterial microbiota is recruited generally from the soil, the profile of microbiota is affected by the roots more than by soil and plants (Tkacz *et al.* 2020). Therefore, vegetation development is hypothesised to increase the chance for bacteria to colonise these wetlands.

We conclude that it is possible for gut bacteria to establish in post-mined wetlands, even though their abundance is variable. In addition, vegetation types rather than mined years influence the occurrence of gut bacteria at various taxonomic levels. This also suggests a hypothesis that mammals in wetlands have an important role in determining the dispersion and immigration of gut bacteria. Wetland restoration after the abandonment of farms attempts to rewild the communities of resident soil microbes and has been reported to succeed in returning microbes (Andras *et al*. 2020). Since the restoration of such disturbed wetlands promotes the recovery of wildlife (Benson *et al*. 2018), the presence of obligate gut bacteria in peat may therefore indicate ecosystem recovery.

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AUTHOR CONTRIBUTIONS

ST and TS designed and prepared the research. RSA and TS conducted OTU measurements and ST conducted statistical analyses. All authors conducted the field work and contributed to the manuscript.

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