Doctoral thesis

Ecological consequences of genetic variation in a foundation species on above- and below-ground communities in the field

（地上部−地下部群集における生態系基盤種の遺伝的変異の影響に関する生態学的研究）

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Chapter 1. General introduction

How ecological communities are shaped by biological interactions is one of the central questions in ecology (Thompson et al., 2001; Webb et al., 2002). Traditionally, many studies have addressed the question under the assumption that conspecific individuals are homogeneous and interchangeable (Bolnick et al., 2011). On the other hand, in evolutionary biology and population genetics, intraspecific genetic variation has long recognized to be common in nature (Charlesworth & Charlesworth, 1978; Charlesworth & Ganders, 1979; Uyenoyama & Feldman, 1980; Wright, 1948). For a few decades, researchers have shown great impacts of intraspecific variation on strength, direction, and outcomes of ecological interactions, such as prey-predator interactions and host–parasite interactions (Moran, 1981; O'Brien & Evermann, 1988; Pimentel & Bellotti, 1976; Service, 1984; Wainhouse & Howell, 1983).

Community and ecosystem genetics

With the increase in the acknowledgement of effects of genetic variation on ecological interactions, a research framework, which is called "community and ecosystem genetics" has been proposed to integrate biological hierarchy from genes to ecosystems (Antonovics, 1992; Mitton, 2006; Whitham et al., 2003). In the studies of community and ecosystem genetics, impacts of genetic variation within a foundation species on the structure of associated communities have received much attention (Whitham et al., 2006; 2008). The foundation species is defined as a species, which has the following characteristics: (i) being locally abundant and regionally common, (ii) having structural or functional characteristics that create a habitat for most of associated species and (iii) modulating core ecosystem processes such as energy and nutrient fluxes or water balance (Ellison et al., 2005; 2010).

In this framework, researchers have provided evidence that genetic variation in a foundation species influences community structure and ecosystem processes. Firstly, natural hybrid zones of two plant species had been commonly used to examine effects of genetic variation in foundation species on shaping arthropod communities (Table 1-1; Dungey et al., 2000; Whitham et al., 1994; Wimp et al., 2004; 2005). Secondly, many studies had begun to experimentally test effects of intraspecific genetic variation and diversity on arthropod communities, using clonal plants (Table 1-1; Crawford et al., 2007; Johnson & Agrawal, 2005; Johnson et al., 2006; Tack et al., 2010). Furthermore, Crutsinger et al. (2006) experimentally demonstrated that greater genetic diversity in *Solidago altissima* supported greater species richness of herbivores but also predators via increase in aboveground primary productivity of the plants. A lot of studies have supported that genetic variation in a plant species affected associated community structures through trophic interactions (Castagneyrol et al., 2012; Crawford et al., 2007; Johnson et al., 2006; Moreira et al., 2012; Moreira & Mooney, 2013).

Relative importance of genetic variation in plants on shaping community structures

Previous community and ecosystem genetics studies have been mainly performed under homogenous environments, using a common garden experiment approach to experimentally test genetic effects (Hersch-Green et al., 2011). Recently, subsequent studies have pay attentions to the relative importance of genetic variation in a foundation species on shaping associated community structures with environmental variation. Several studies used multiple common gardens in different locations to investigate the relative importance of the genetic variation on shaping community structures, compared to different environments (Fritz, 1990; Quiring & Butterworth, 1994; Rossi & Stiling,

1998; Stiling, 1994; Stiling & Bowdish, 2000; Stiling & Rossi, 1995, 1996; Strauss, 1990; Tack et al., 2010). These experiments demonstrated that different locations were likely to be more important for shaping insect communities than genetic variation in plant species. However, Johnson & Agrawal (2005) pointed out scale-dependence in the relative importance between genetic variation in the plant and environmental conditions. To test the scale-dependence in the effects of genetic variation on shaping community structures, Johnson & Agrawal (2005) compared effects of plant genotypes on associated communities among and within common gardens. The relative importance of genetic variation in plant species on arthropod communities depended on spatial scales. Silfver et al. (2014) also supported this notion.

Unresolved issues

Nevertheless, the knowledge of the importance of genetic variation in foundation species on shaping associated communities in natural ecosystems is still limited because of the two following reasons. First, the common garden approach was usually employed to exclude environmental and spatial variability (Hersch-Green et al., 2011). The reduction in the abiotic variability may lead to overestimation of the importance of genetic variation in shaping associated communities even if multiple common gardens are applied (Gugerli et al., 2013; Hersch-Green et al., 2011). Therefore, the importance of genetic variation in a plant species on assembly of associated community is still unclear in a natural ecosystem.

Second, no studies have focused on the genetic effects of host plants on mutualistic interaction with microsymbionts in rhizosphere under natural environments despite a lot of the knowledge which diverse microsymbionts exist in rhizosphere

(Bahram et al., 2011; Ben Tekaya et al., 2018; Jumpponen et al., 2010; Mishra et al., 2015; Moawad & Schmidt, 1987; Põlme et al., 2014; Shiraishi et al., 2011; Toju et al., 2014). Many recent studies have provided evidence that genetic variation in a plant species influences belowground communities, as well as the aboveground communities (Evans et al., 2016; Genung et al., 2013; Lamit et al., 2015; Lankau, 2011; Schweitzer et al., 2008; 2014; 2018; terHorst & Zee, 2016). For examples, Lankau (2011) found that intraspecific variation in allelochemical, such as glucosinolate and flavonoids, of *Allaria petiolata* influenced belowground community composition, including bacteria and fungi. Similarly, Genung et al. (2013) reported that genetic variation in *Solidago altissima* affected soil microbial communities through leaf litter decomposition. These studies suggested that integrating of effects of genetic variation in a plant for above- and below-ground communities is needed to understand the impacts of genetic variation in a plant species on natural ecosystems. However, the previous studies have never focused on plant–microbe mutualistic interactions while the studies paid attention to free-living organism communities in soils. Furthermore, plant-microbe mutualism can also influence community structures of associated arthropods mediated by phenotypic variation in host plants (Ballhorn et al., 2013; 2017; Dean et al., 2009; Godschalx et al., 2017; Katayama et al., 2011; 2014; Thamer et al., 2011; Whitaker et al., 2014). Therefore, incorporating community composition of mutualistic symbionts within plant individuals is required toward an understanding the importance of genetic variation for plants in associated community dynamics in natural ecosystems.

Goals of this thesis

The objective in this thesis is to reveal impacts of genetic variation in a foundation species

on associated community structure in a natural ecosystem. In order to reach the objective, I elucidate the impacts of genomic variation in a foundation species on above- and below-ground associated communities in a natural ecosystem. In particular, I addressed the four following questions: (1) are distinct associated communities shaped on genetically distant host trees, (2) is a host tree exposed by high genetic diversity of mutualistic bacteria in a natural habitat, (3) how is mutualistic community in a host individual constructed from surrounding environments, and (4) does symbiont composition in a host individual affect growth, survival and preference of a herbivorous insect? I focused on the alder, *Alnus hirsuta* Spach (Betulaceae). The alder species, which is a deciduous broadleaf tree and an early successional species, is widely distributed in temperate riparian forests of Japan, northeastern China, Korea, and Russia. Alder trees have the following characteristics as foundation species in a riparian forest ecosystem (Ellison et al., 2005; 2010): (1) they are a dominant species in early succession forests, (2) they support diverse arthropod species (Kagiya et al., 2018; Nyeko et al., 2002), and (3) they are actinorhizal species able to form partnerships with nitrogen-fixing actinobacteria, *Frankia* spp. (Frankiaceae) forming nodules in their roots, which seem to greatly affect ecosystem processes such as nutrient cycling.

This thesis includes five chapters, except for this general introduction. In Chapter 2, I estimated the relative importance of natural genomic variation of the foundation species on shaping associated arthropod communities in a natural habitat, using numerous numbers of genome-wide markers with next-generation sequencing (NGS). In Chapter 3, I investigated spatial structure in genetic diversity and composition of mutualistic bacteria in a forest scale. In Chapter 4, I comprehensively explored genetic composition of mutualistic bacteria, which interact with host individuals, and are present in rhizosphere

soils and host-absent soils, by metabarcoding using NGS. Subsequently, I compared structures of the three mutualist communities to reveal a process of shaping mutualistic bacteria community on plants. In Chapter 5, I conducted experiments using natural alder–*Frankia* symbiosis to test whether natural genetic composition of mutualistic bacteria in the host individual affected growth, survival and preference of an herbivorous insect. In Chapter 6, I synthesize these studies to propose the concept integrating plants–bacteria mutualism into community and ecosystem genetics.

Table 1-1. Principal studies in the framework of "community and ecosystem genetics".

Studies	Design	Plant organisms		Arthropods	
Whitham et al. (1994)	FI	**Plants**	*Eucalyptus risdonii* *E. amygdalina*	**Organisms**	Arthropod community
		Genetics	Interspecific hybrids		
		Identify	Phenotype	**Parameters**	Abundance, Species richness
Floate et al.(1997)	FI	**Plants**	*Populus fremontii* *P. angustifolia* *P. balsamifera* *P. trichocarpa* *P. deltoides*	**Organisms**	*Pemphigus betae*
		Genetics	Hybrids populations		
		Identify	RFLP	**Parameters**	Gall number Relative abundance
Dungey et al. (2000)	SCE	**Plants**	*Eucalyptus risdonii* *E. amygdalina*	**Organisms**	Arthropod community
		Genetics	Interspecific hybrids		
		Identify	Phenotype	**Parameters**	Species richness, Species composition
Wimp et al.(2004)	SCE & FI	**Plants**	*Populus fremontii* *P. angustifolia*	**Organisms**	Arthropod community
		Genetics	Genetic variation, Genetic diversity		
		Identify	RFLP, AFLP	**Parameters**	Species composition, Species diversity
Wimp et al.(2005)	SCE & FI	**Plants**	*Populus fremontii* *P. angustifolia*	**Organisms**	Arthropod community
		Genetics	Genetic variation		
		Identify	RFLP	**Parameters**	Species composition
Bailey et al.(2006)	SCE	**Plants**	*Populus angustifolia*	**Organisms**	*Pemphigus betae* *Pheucticus melanocephalus* *Turdus migratorius* *Poecile atricapilla*
		Genetics	Genetic variation		
		Identify	RFLP	**Parameters**	Gall number, Attack number on gall
Crutsinger et al. (2006)	SCE	**Plants**	*Solidago altissima*	**Organisms**	Arthropod community
		Genetics	Genetic diversity		
		Identify	AFLP	**Parameters**	Species richness
Crutsinger et al. (2014)	SCE	**Plants**	*Baccharis pilularis*	**Organisms**	Arthropod community
		Genetics	Genetic variation		Species richness, Abundance,
		Identify	Phenotype	**Parameters**	Species composition
Crawford et al. (2007)	SCE	**Plants**	*Solidago altissima*	**Organisms**	Arthropod community
		Genetics	Genetic diversity		Species richness, Abundance,
		Identify	Clone	**Parameters**	Species composition
Moreira & Mooney (2013)	SCE	**Plants**	*Baccharis salicifolia*	**Organisms**	*Aphis gossypii* *Linepithema humile* *Braconidae*
		Genetics	Genetic diversity		
		Identify	Clone	**Parameters**	Abundance
Johnson et al. (2006)	MCE	**Plants**	*Oenothera biennnis*	**Organisms**	Arthropod community
		Genetics	Genetic diversity		
		Identify	Clone	**Parameters**	Species richness, Abundance
Castagneyrol et al. (2012)	SCE	**Plants**	*Quercus robur*	**Organisms**	Arthropod community
		Genetics	Genotype, Genetic diversity,	**Parameters**	Herbivory damage

			Relatedness		
		Identify	Microsatellite		
Relative importance with spatial scale					
		Plants	*Oenothera biennis*	**Organisms**	Arthropod community
		Genetics	Genotype		Species diversity, Evenness, Species richness, Abundance,
Johnson & Agrawal (2005)	MCE	**Identify**	Clone	**Parameters**	Biomass
		Plants	*Quercus robur*	**Organisms**	Arthropod community
		Genetics	Genotype		Species richness,
Tack et al. (2010)	MCE	**Identify**	Clone	**Parameters**	Abundance
		Plants	*Betula pendula*	**Organisms**	Arthropod community
		Genetics	Genotype		Species composition,
Silfver et al. (2014)	MCE	**Identify**	Clone	**Parameters**	Abundance
Relative importance with plant species diversity					
		Plants	*Oenothera biennis*	**Organisms**	Arthropod community
		Genetics	Genetic diversity		Species richness,
Cook-Patton et al. (2011)	SCE	**Identify**	Microsatellite	**Parameters**	Species composition
		Plants	*Ammophila breviligulata*	**Organisms**	Arthropod community
		Genetics	Genetic diversity		Species composition, Abundance, Species richness,
Crawford & Rudgers (2013)	SCE	**Identify**	Population	**Parameters**	Evenness
					Hypsipyla grandella *Phyllocnistis meliacella* Cicadellidae
		Plants	*Swietenia macrophylla*	**Organisms**	Araneae
		Genetics	Genetic diversity		
Abdala-Roberts et al. (2015)	SCE	**Identify**	Mother tree	**Parameters**	Abundance, Herbivory damage
Relative importance in a field					
		Plants	*Quercus petraea* *Q. robur*	**Organisms**	Arthropod community
		Genetics	Genetic distance		
Gossner et al. (2015)	FI	**Identify**	Microsatellite	**Parameters**	Community dissimilarity
		Plants	*Quercus robur*	**Organisms**	Arthropod community
		Genetics	Genetic distance		
Pohjanmies et al. (2015)	FI	**Identify**	Microsatellite	**Parameters**	Community dissimilarity

Abbreviations in the table
FI: Field investigation
SCE: Single common garden experiment
MCE: Multiple common garden experiment

Chapter 2. Does genomic variation in a foundation species predict arthropod community structure in a riparian forest?

Introduction

Researchers have long been interested in how ecological communities are shaped by abiotic and biotic factors, including interspecific interactions (Thompson et al. 2001; Webb et al. 2002). Traditionally, many studies on community ecology and species interactions have implicitly assumed that all conspecific individuals are homogeneous and interchangeable (Bolnick et al. 2011). However, intraspecific trait variation is common in nature, and growing evidence has shown that genetic variation within species affects population dynamics, community properties of associated organisms, and ecosystem processes (Fritz and Price 1988; Whitham et al. 2003; Ellers 2009). For example, genetic variation in a host plant species affects a population growth rate of an herbivorous insect (Utsumi et al. 2011), species richness and abundance of plant-associated arthropods (Fritz et al. 1987; Crutsinger et al. 2006; Johnson et al. 2006), and litter decomposition (Schweitzer et al. 2005).

On the basis of the increasing recognition of the link between plant genes and associated communities, Bangert et al. (2006) proposed a genetic similarity rule: plants with more similar genotypes will have more similar traits (e.g., leaf chemistry), thus supporting more similar arthropod communities. This rule may be the key to understand how communities are shaped in a complex ecosystem.

To test the genetic similarity rule, the impacts of genetic variation within a foundation species on the structure of associated communities have received much attention (Whitham et al. 2006, 2008). The foundation species is defined as a species, which has the following characteristics: 1) being locally abundant and regionally common, 2) having structural or functional characteristics that create a habitat for most of associated species, and 3) modulating core ecosystem processes such as energy and

nutrient fluxes or water balance (Ellison et al. 2005, 2010). The evidence that genetic variation within a foundation species affects arthropod communities has been provided by previous studies (Fritz and Price 1988; Dungey et al. 2000; Hochwender and Fritz 2004; Shuster et al. 2006; Barbour et al. 2009; Zytynska et al. 2011; Barton et al. 2015).

In this context, recently, several studies have paid attention to the relative importance of genetic variation in shaping associated communities in comparison with other ecological forces. Studies using geographically distant common gardens reported that spatial locations of populations were more important for species richness than genetic variation in tree species (Tack et al. 2010; Tack and Roslin 2011). Johnson and Agrawal (2005) and Silfver et al. (2014) compared the influence of genetic variation in a plant species with the effects of environmental factors (i.e., effects of different common gardens) on arthropod communities. These studies suggested that both genetic and environmental factors were important for determining community structure of herbivore arthropods. Conversely, Evans et al. (2012) reported that genetic variation in poplar trees critically influenced mite population size for eight years. In comparing surrounding local flora of a focal plant species, Abdala-Roberts et al. (2015) indicated that the effects of genetic diversity of a focal woody plant species were less important than surrounding plant species diversity of a focal plant individual on arthropod community structure. Therefore, the relative importance of genetic variation in shaping community structure is still under debate. Because numerous biotic interactions and abiotic factors, including temperature and precipitation, may simultaneously contribute to shape arthropod community structure in natural ecosystems (Hunter and Price 1992; Hodkinson 2005), I should test the genetic similarity rule under a complex field condition.

To test the genetic similarity rule in the wild, two lines of limitations of previous

studies have begun to be acknowledged (Hersch-Green et al. 2011; Gugerli et al. 2013). First, majority of these studies employed the common garden approach to exclude environmental and spatial variability and to set genotypic replicates. The reduction in environmental and spatial variability may lead to overestimating the importance of genetic variation in shaping associated communities. Moreover, the arbitral selection of genotypes used in a common garden experiment may result in an inaccurate estimation of the importance of genetic variation in a natural ecosystem (Hersch-Green et al. 2011). Only a few studies have investigated the genetic effects on foundation species under natural heterogeneous environmental conditions in the field (Gossner et al. 2015; Pohjanmies et al. 2015). Second, most studies used only a few microsatellite markers or amplified fragment length polymorphism (AFLP) markers to quantify genetic variation. Community genetics requires at least the description of genetic variation corresponding to differences in traits of host plants, which are relevant for shaping associated community structure (Gugerli et al. 2013). However, the genetic variation estimated by such markers is often unlikely to reflect those trait variations in wild populations. The inconsistent patterns between plant genetics and associated communities can be resulted from genetic markers.

Thus, in this study, I focused on natural genetic variation of trees in a natural habitat, describing genetic variation by numerous numbers of genome-wide markers with next-generation sequencing (NGS). This technique allows to accurately estimate high-resolution genetic variation within and/or among populations. I investigated the impact of genetic variation in a foundation species (*Alnus hirsuta* Turcz. ex Rupr.) on community structure of associated arthropods in a natural ecosystem. *Alnus* trees have the characteristics of a foundation tree species: (1) a dominant species in early succession

forests, (2) supporting diverse arthropod species (Nyeko et al. 2002), and (3) a symbiosis with nitrogen-fixing microbes in roots that seems to greatly affect ecosystem processes, such as nutrient cycling. In particular, I address the following questions: (1) Does the natural genetic variation within a foundation species explain associated arthropod communities in wild? (2) To what extent does genetic variation in a foundation species explain the variation of arthropod communities in comparison with biotic and abiotic factors, such as spatial locations and environmental conditions? (3) Does the genetic variation within a foundation species predict year-to-year changes in arthropod community structure? (4) Which functional groups of arthropods respond more sensitively to genetic variation in a foundation species?

Materials & Methods

Study organism and sites

Alnus hirsuta is a deciduous broad-leaf tree and an early successional species. It is widely distributed in temperate riparian forests in Japan, northeastern China, Korea, and Russia. A wide variety of arthropod species, including polyphagous and monophagous herbivore species, and their predators, colonize on *A. hirsuta*.

Our study sites are located in and around Uryu experimental forest (44°03'-29'N, 142°01'-20'E) of Hokkaido University in northern Hokkaido, Japan. This experimental forest area is approximately 25,000 ha. I used 12 surveyed riparian sites (Table 2-1). *Salix sachalinensis* and *Betula platyphylla* are common in these sites, aside from *A. hirsuta*.

Genetic characterization of A. hirsuta trees

To characterize genetic variation of alder trees, I developed a genome-wide

database of the alders in my study sites. I collected leaves from 276 alders across these sites from July to September in 2013. The leaves were immediately stored in a freezer at -20 °C until genomic DNA extraction.

The genomic DNA of alders was extracted by NucleoSpin PlantII kit (MACHEREY-NAGEL, Düren, Germany). DNA concentration was measured by Qubit® 2.0 Fluorometer (Invitrogen, Darmstadt, Germany) with a DNA-specific probe.

To discover single nucleotide polymorphisms (SNPs), Double-digested restriction-site-associated DNA sequencing (ddRADseq; Peterson et al. 2012) was conducted using HiSeq system. RAD libraries were prepared with two restriction enzymes BglII and EcoRI-HF. Details of the protocol, reagents and oligo-nucleotide sequences for the RAD-Seq library preparation were described previously (Sakaguchi et al. 2015). Low quality reads that were obtained from RAD-Seq were removed with the Trimmomatic software (parameters used: LEADING:19, TLRAILING:19, SLIDINGWINDOW:30:20, AVGQUAL:20, MINILEN:51). I used the denovo_map.pl pipeline provided by Stacks software to call SNPs directly from the processed data (parameters used: -m 3 -M 2 -n 0; Catchen et al. 2013). I removed SNPs whose depth of coverage was lower than 10, missing rate was more than 75% of the samples, and minor allele frequency (MAF) was lower than 1%. Then, I analyzed for one SNP per one locus to avoid linkage disequilibrium and 216 trees were genotyped using Stacks software. Raw sequence data were deposited at DDBJ Sequence Read Archive (DRA) with accession number DRA006178.

Arthropod communities on alders

From the 216 alder trees, I randomly selected 85 alder trees found in my 12 study

sites. These trees were mature and their DBH was 18.44 ± 9.54cm (mean ± SD). To investigate alder-associated arthropod communities, I performed a field survey in 2014 and 2015. I collected arthropods from each tree crown in July and August, using a butterfly net (5m in pole length and 40cm in net diameter). Arthropods, such as leafhoppers (Hemiptera), leaf beetles (Coleoptera), wasps (Hymenoptera), and spiders (Araneae), were assessed for each alder individual by sweeping in one minute twice in both years. These arthropod species were identified, and the numbers of individuals were recorded.

To estimate the community dissimilarity of arthropods on the tree crown, Morisita-Horn index was calculated with R, using the vegan 2.3-2 package (Oksanen et al. 2015). Morisita-Horn index is a quantitative similarity index independent of sample size. I also used Bray-Curtis index, which is one of the most common similarity indices in community ecology to strengthen the validity of results based on Morisita-Horn dissimilarity. The total number of individuals of each species in each site was used for community dataset at the site level. The dataset was standardized to a unified scale [0; 1], dividing by the maximum number of each species. The reason for this standardization is that variances of the numbers are greatly unequal among the species, especially among different functional groups (functional groups are defined in the next paragraph).

For further analyses, arthropods were classified to five functional groups (Table 2-3). “Chewer group” comprises herbivores such as leaf beetles (Coleoptera) and caterpillars (Lepidoptera) that externally feed on leaves. “Sap-feeder group” is another herbivore group that feed on phloem sap with a stylet, representing hemipteran aphids and leafhoppers (Hemiptera). “Spider group” is composed of Araneae species. “Carnivore group” comprises predacious insects, such as parasitoids wasps (Hymenoptera) and

predatory shield bugs (Hemiptera). The remaining arthropod species that are not included in the above four functional groups are referred to as “Other group”. The dataset of the total number of individuals of each species was divided among the functional groups, and then compositional dissimilarity of each functional group was calculated, using Morisita-Horn index.

To consider spatial and temporal autocorrelations in the arthropod community structure, I calculated spatial distance and temporal distance between sites. I used the mean survey date of alder individuals at each site as the temporal difference at the site level, which was represented by Euclidian distance. Spatial distance was calculated based on latitude and longitude of each alder using the Great Circle distance method. Centroid location as the averaged latitude and longitude at each site was used to calculate the spatial distance at the site level. The spatial distance and the temporal difference were calculated using the R package sp 1.2-1 (Pebesma and Bivand 2005). In addition, I did not only consider spatial distance as direct geographical distances but also as river path distances because of a dispersal possibility of arthropods associated alders along a river network. The nearest spatial distance between pairs of sites through the river lines was calculated by network analysis with ArcGIS 10.4.

Leaf traits of alders

To measure leaf traits of the focal alders, I randomly collected leaves (20 leaves from 5 shoots in 2014 and 6 leaves from 3 shoots in 2015) from each individual during the same period of sampling arthropods, using high branch-pruning scissors. The collected leaves were immediately stored in a cool box and transferred to a refrigerator in a laboratory at that day. All leaves were scanned with a digital scanner and their wet

weight was measured within 24h from the sampling day. The leaf area was measured from the digital image, using Image J version 1.50 (Rasband 1997-2012). The leaves were oven-dried at 60 °C for 48 h, and then their dry weight was measured. The water content of each leaf was calculated by subtracting dry weight from wet weight.

Part of the dried leaves (n = 170 in 2014, n = 255 in 2015) was ground with a mixer mill (MM200: Retsch, Retsch-Alley, Haan, Germany). For grinding the leaves, the heaviest dry weight of leaves from each shoot (two of five shoots in 2014, three shoots in 2015) was selected. I analyzed the total carbon and nitrogen concentration of leaves, using a CHNS/O analyzer (PE2400II: ParkinElmer, Waltham, Massachusetts, USA). Then, carbon-nitrogen (C:N) ratio was calculated.

To estimate the dissimilarity of leaf traits among sites, Mahalanobis distance was calculated, based on site-averaged six leaf traits (i.e., leaf area, dry weight, water content, specific leaf area (SLA), leaf mass per area (LMA), and C:N ratio) using the R package StatMatch 1.2.3 (D'Orazio 2015).

Tree communities surrounding alders

To compare relative impacts of genetic variation in host trees and effects of local environments on organizing arthropod communities, I also assessed the community structure of trees. The local tree communities seem to represent local environmental conditions because local tree communities are likely to act as an environmental filter for inhabiting arthropods and a source of colonizer arthropods onto alders, which may regulate arthropod community structure on the focal alder trees. I recorded all trees (DBH > 10cm) within a radius of five meters from a focal alder individual (Table 2-4). To represent such local flora functions, not only the species identity but also their

phylogenetic relationships were also considered. First, I calculated the phylogenetic distance among observed tree species based on Zanne et al. (2014) using the software Phylomatic (http://phylodiversity.net/phylomatic). Second, phylogenetic community dissimilarity (PCD) of site-level tree communities was provided on the basis of this phylogenetic distance, using the R package picante 1.6-2 (Kembel et al. 2010).

Statistical analysis

The dissimilarity matrices of the arthropod communities were analyzed with the genetic distance, leaf traits dissimilarity, PCD, spatial distances, and temporal difference by Mantel tests and partial Mantel tests. These tests were performed with 9999 permutations. To estimate the genetic distance among-site alder populations, I calculated Nei's genetic distance (Nei 1978), based on obtained SNPs by RAD-Seq.

Generalized dissimilarity modelling (GDM) (Ferrier et al. 2007) was performed to quantify the relative contribution of genetic distance and other factors in explaining the spatial patterns of community dissimilarity. GDM is an I-spline-based extension of matrix regression that accounts for nonlinearities between explanatory variables and community dissimilarity. As GDM uses flexible splines, it is capable of fitting non-linearity relationships between community dissimilarity and environmental variables (Ferrier et al. 2007). The community dissimilarities in each year, taken as Morisita-Horn dissimilarities, were used for GDM analyses. GDM models for the species in 2014 and 2015 included Nei's genetic distance, PCD, spatial distance, and temporal distance as predictors.

To test whether the genetic distance of alders could explain the dissimilarity of arthropod communities without other factors, I estimated unique and shared contribution of each predictor to the total deviance explained by partitioning the predictors with GDM

models (Fitzpatrick et al. 2013). To calculate the unique contribution of genetic distance and other factors, the deviance explained of the model removed each predictor was subtracted from the deviance explained of the full model. The results were converted into percentages where the deviance explained of the full model was 100 %. To calculate the shared contributions of both predictors, a value of deviance explained of the model that was fitted by both predictors was subtracted unique contributions of each predictor.

To analyze the contribution of genetic variation in the foundation species to year-to-year changes in associated communities, I quantified the year-to-year changes in arthropod communities throughout the two years, employing non-metric multidimensional scaling (NMDS). NMDS in a three-dimensional space was performed with Morisita-Horn dissimilarity using vegan package. The stress of performed NMDS was 0.15, which indicates a good representation of the data in three-dimensional ordination plot. For scores along each axis of the three NMDS axes, the scores between the two years within each site were subtracted. The three-dimensional dataset of the year-to-year changes was obtained, and then yearly variation dissimilarity among the sites was calculated as Euclidian distance using the dataset.

To examine which the functional groups responded to genetic variation in the host trees, the correlation between compositional dissimilarity of each functional group and Nei's genetic distance was analyzed, using Mantel and partial Mantel tests.

All the above statistical analyses were performed using the R software version 3.2.3 (R Core Team 2015). NMDS, Mantel tests and partial Mantel tests were performed with vegan package. GDM was performed, using gdm 1.1.7 package (Manion et al. 2015).

Moreover, structural equation modeling (SEM) was performed to better understanding how genetic distance of alders influenced herbivore and predator

communities. In this modeling, I focused on genetic distance of alders, leaf dissimilarity, and each abundance of arthropod functional groups, and primarily aimed to examine causational relationships from genetics to communities (i.e., genetic effect via focal leaf traits, bottom-up effects of plants on predators mediated through herbivores, and top-down effects on herbivore through predators). To manage distance matrix data as variables in SEM analyses, I performed a principal coordinate analysis (PCoA), using the cmdscale function in the R software. PCoA takes a symmetric matrix of distances of any type among replicates and produces corresponding coordinates for each replicate which, in the full-dimensional principal coordinate space, preserve the original distances calculated among replicates (Gower 1966; Legendre and Anderson 1999). Thus, as genetic and leaf trait variables in SEM, I used scores of the first two or three PCoA axes, which showed more than 80% cumulative contribution in genetic distance matrix and leaf trait dissimilarity, respectively. For the abundance of four functional groups (chewer, sap-feeder, spider, and carnivore group), I used log-transformed average abundance per a tree of each group. I proposed hypothetical models (Fig. 2-5a, b), considering following causational paths: (1) from genetics to leaf traits, (2) from leaf traits to all functional groups, (3) from genetics to all functional groups because gene-based non-focal traits might affect arthropods. In the bottom-up model, I added paths from the chewer and the sap-feeder to the carnivore and the spider, and also added a path from the carnivore to the spider to consider predation of carnivore insects by spiders (Fig. 2-5a). In the top-down model, opposite directional paths between the functional groups were considered (Fig. 2-5b).

Alternative structural equation models were generated by modifying the full causal model using a backward stepwise elimination process in which non-significant

relationships were removed from the full model (starting with the least significant path) until all relationships within the model were significant (Grace 2006). Chi-square lack-of-fit test was performed to examine the fit of the alternative structural equation model. Proposed models with P values > 0.05 were accepted as a good fit to the data (Grace 2006). The best model was finally selected based on goodness-of-fit measures (comparative fit index: CFI, root mean square error of approximation: RMSEA, and Akaike information criterion: AIC). The strength of indirect effects was calculated by multiplying the path coefficients along the individual paths mediated by associated variables in the best models. The strength of total effects was calculated by adding direct effects to indirect effects. All SEM analyses were performed using AMOS 5.0.

Results

Genetic characterization of alders

A total of 166.5 million of 51-bp raw single-end reads were obtained by RAD-Seq. I obtained 424,205 contigs by *de novo* assembly of the reads. 50,524 of 424,205 contigs included SNPs. To extract high-quality SNP markers, I applied a conservative filter to the 50,524 contigs with SNP. A set of 1077 high-quality SNP markers were obtained after above filtering and were used to genotype 216 alder trees.

Arthropod communities on alders

I collected 2078 individuals (24.45 ± 15.10 per tree, mean ± SD) belonging to 74 species (10.04 ± 3.68) in 2014, and 3053 individuals (35.92 ± 29.91) belonging to 97 species (10.07 ± 4.17) in 2015. In total, 58 families were collected (Table 2-3).

A relationship between genetic variation of host plants and arthropod communities

I found significant correlations between the genetic distance of *A. hirsuta* and the community dissimilarity of arthropods in both years (Fig. 2-1). These correlations remained significant, even when effects of other factors, such as leaf traits, PCD, spatial distance or temporal distance, were corrected using partial Mantel tests (Table 2-2). The Bray-Curtis dissimilarity also provided consistent results (Table 2-2).

Explanatory power of genetic distance on community dissimilarity

Using the GDM approach, the genetic distance of alders and other factors explained 63.39% and 36.52% of deviance in the community dissimilarity of arthropods in 2014 and 2015, respectively. Where the deviance explained by the model with genetic distance and other factors was 100%, genetic distance uniquely explained 87.04% of deviance in 2014. Other predictors explained 6.22% of the deviance. In 2015, 60.92% and 20.53% of the deviance was explained by genetic distance and other factors, respectively (Fig. 2-2).

G*enetic variation in host plants vs. year-to-year changes in communities*

A significant correlation was found between the genetic distance of *A. hirsuta* and the year-to-year changes in the dissimilarity of arthropod communities (Fig. 2-3, r = 0.669, P = 0.012). The correlation between the genetic distance of *A. hirsuta* and the vector difference of year-to-year changes in the community dissimilarity remained significant even when the effects of other factors were corrected with partial Mantel tests (Table 2-7).

Responses of functional groups to genetic variation in host plants

For the analysis of the five functional groups, the compositional dissimilarity of the carnivore group was strongly and significantly correlated with the genetic distance of *A. hirsuta* in both years (Fig. 2-4d, 2014: $r = 0.640$, $P = 0.008$; 2015: $r = 0.401$, $P = 0.036$). In 2014, the chewer and the spider groups were also correlated with genetic distance (Fig. 2-4a: $r = 0.372$, $P = 0.010$; Fig. 2-4c: $r = 0.347$, $P = 0.024$). Partial Mantel tests that corrected other predictors provided consistent results (Table 2-5).

I performed SEM analysis using dataset in 2014 (Fig. 2-7 and Table 2-8) and 2015 (Fig. 2-5 and Table 2-9), respectively. The results in 2015 were shown in the main text because patterns in both years substantially consistent. The best models of both the bottom-up and top-down hypotheses were very similarly acceptable based on goodness-of-fit measures (CFI, RMSEA, and AIC; Fig. 2-5c, d). Both models similarly showed significant links from genetic variation to arthropods through focal leaf traits as well as significant direct paths between genetics and arthropods (Fig. 2-5c, d). Furthermore, the strengths of the total effects of non-focal traits on each arthropod group (i.e., total effects from genetics to arthropods without paths through focal traits) tended to be as strong as the total effects of focal leaf traits on arthropod groups (i.e., effects from traits to arthropods; Table 2-9). These results indicated that arthropod communities were shaped by both genetically-based, focal and non-focal leaf traits of alders.

In addition, similar goodness-of-fit measure scores between the best models of the bottom-up and top-down hypotheses suggested that both significant bottom-up and top-down interactions between the functional groups would contribute to shaping overall community structure. The total effects of genetics on the abundance of each functional group were not largely different between both the bottom-up and top-down models (Table

2-9). These suggested that genetic effects of alders would propagate to community members through both the bottom-up and the top-down interactions.

Discussion

This study clearly demonstrated that natural genetic variation in the foundation tree species effectively predicted the differences in associated arthropod community structure in wild in both of the two years. Furthermore, I found that genetic variation in the foundation species did not only predict spatial structure of arthropod communities but also year-to-year temporal changes in arthropod communities. Among the functional groups, counterintuitively carnivore insects responded the most to genetic variation in the foundation species rather than herbivorous groups.

Relationship between genetic variation in a foundation species and arthropod communities in wild

Although only a few previous studies investigated the relationships between natural genetic variation in foundation species and community structure of arthropods in the field, inconsistent results were reported. Gossner et al. (2015) found that insect communities were not significantly correlated with genetic variation in two oak species, whereas Pohjanmies et al. (2015) detected a significant correlation between genetic variation in an oak species and herbivore communities. These studies used only a few microsatellite markers to characterize genotypes of tree species, and only focused on limited insect groups. By contrast, this study represented genetic variation in a tree species based on thousands of genome-wide SNP markers and found a profound association between tree genetics and associated arthropod communities across two years. To my

knowledge, this study is the first that to apply thousands of SNPs for linking tree genetics with associated communities.

Importantly, correlograms of the community dissimilarity of arthropods and the genetic distance of alders along spatial distance classes showed different patterns (Fig. 2-6). This outcome suggests that the observed relationship between tree genetics and arthropod communities was not likely to appear by spatial autocorrelations but by genetic characteristics. In addition, I examined not only simple geographic distance but also the least distance through river lines, as alders and associated arthropods may disperse through a riparian habitat rather than through a straight geographic distance. However, no significant relationship was detected (Table 2-2). Therefore, my results reject the spatial autocorrelation effects on the relationship between alder genetics and arthropods community structure.

Because some SNPs may be closely associated with phenotypic variation, which critically affects arthropod communities and experiences past and ongoing natural selection, high-density SNPs facilitates the study of the genetic similarity rule among a wide variety of organisms even when reference genome sequences are unknown. In this study, I did not detect loci with a clear signal of selection within this sampling scale by bayescan approach ($q > 0.05$, data not shown). This may suggest that these SNPs would not be under consistent selection in the among-site level. However, SNPs variation certainly associated with measured leaf traits and arthropods (Fig. 2-5, 7; Table 2-6). This is likely to be because genetic distance estimated by the SNPs would well represent relatedness between alder individuals, which in turn, detected close relationships among genetics, traits, and arthropod communities. Nevertheless, selection could also play a role for realizing the genetic similarity rule. Frequency-dependent selection and temporally-

spatially variable selection within a site scale may prevent among-site genetic differentiation and/or selective sweep in SNP markers, which associated with adaptive genes. For example, Sato et al. (2017) suggested that optimal foraging behavior of by an herbivore population could generate negative frequency-dependent selection between trichome-producing and trichomeless plants, resulting in the maintenance of polymorphism of the plant defensive traits in a local site scale. In this case, plant genetics can regulate plant-herbivore interactions and strongly influence herbivore abundance at each site and at each time, though significant selection signals may not be detected among sites but may be found in a much finer scale. Toward a more accurate understanding of genetic effects, a deeper genome-wide analysis and association study related to ecological communities across several spatial scales should be fruitful in future studies.

Relative importance of genetic variation to predict arthropod communities

The GDM approach clearly indicated that genetic variation between the foundation species was the most important predictor of structure of arthropod communities. Some studies tested the relative importance of genetic variation in foundation species on arthropod community structure, comparing with other factors such as spatial locations or environmental conditions (Tack et al. 2010; Tack and Roslin 2011; Silfver et al. 2014; Evans et al. 2012; Gossner et al. 2015; Pohjanmies et al. 2015). However, no studies have thoroughly quantified the relative importance of genetic variation in comparison with multiple scenarios of spatial, temporal and other local biological effects on shaping community of associated arthropods in natural forest ecosystems. According to partitioning predictors, I found a large proportion of unique contribution of genetic variation, and small shared contribution between genetic distance

and other factors. This result clearly indicated that host plant genetic variation primarily determined the structure of associated arthropods, and that a small fraction would be interactively affected by genetics of a foundation species and the environmental factors. However, the degree of unique contribution of genetic distance was slightly different between 2014 and 2015 (Fig. 2-2). Similarly, Silfver et al. (2014) suggested that the relative importance of effects of genetic variation and environmental condition on the structure of arthropod community was variable between years. The relative importance of genetic variation in host species on associated community structure may vary with yearly variation in climate. For example, monthly precipitation in July in 2015 was twice as much as that in 2014. Thus, this climatic difference could affect arthropod composition on tree crown. Nevertheless, genetic variation in a host plant was consistently important in predicting community structure of arthropods in the field.

This study could not remove the possibility that other unmeasured local environmental factors could underlie the community consequences of plant genetic variation. However, note that genetic variation within a foundation species explained community structure of associated arthropods at a relatively large spatial scale across two years rather than spatial location, survey date difference and surrounding tree communities. Therefore, current community structure of arthropods was predicted effectively from genetic structure in a foundation species even if there was such an underlying correlation.

Furthermore, although community structure of associated arthropods varied between the two years, the extent of year-to-year changes was also explained by genetic similarity (Fig. 2-3). Year-to-year changes in communities were likely to arise from interactions between each of community members and biotic/abiotic factors. Thus,

genetic component of a foundation species could influence the subsequent interactions regulating year-to-year population dynamics of community members. My finding suggests that genetic variation within a foundation species does not only explain the snapshot of community structure but may also be important in temporal community dynamics. Future studies should pay attention to the link between temporal community dynamics and plant genetics. It will further provide new insights to combine community ecology and evolutionary biology.

How arthropods respond to genetic variation?

As proposed by the genetic similarity rule, I found a positive relationship between leaf trait distance and community dissimilarity in 2014 and 2015 (Table 2-2), respectively, as well as a positive correlation between genetic distance and leaf trait distance in 2014 but not in 2015 (Table 2-6). In addition, SEM analysis indicated the significant relationships between genetic variation in alders and the abundance of arthropods via variation in leaf traits (Fig. 2-5, 7). These findings could support the importance of the genetic similarity rule for structuring arthropod communities even in the field condition. However, partial Mantel test and SEM suggested that non-focal, genetically-based traits would also influence community structure of arthropods. Partial Mantel test represented that the correlations between genetic distance and community dissimilarity remained significant even when the effect of leaf traits was removed (Table 2-2). This result was supported by SEM results that significant direct effects of genetics in alders to the abundance of arthropods without focal traits were found (Table 2-8 and S7). Therefore, community structure of arthropods is more likely to be affected not only by measured leaf traits (e.g., carbon, nitrogen, water content, and LMA) but also by other

genetic-based plant traits such as secondary metabolites and volatile organic compounds (VOC). This suggestion was at least partially supported by Tscharntke et al. (2001) that chemical compounds, including blends of volatiles with terpenes, within *Alnus* trees affected the abundance of phytophagous and entomophagous arthropods.

From SEM analyses, the genetic effects of the foundation tree can influence arthropods in two ways: (1) direct effect on carnivores and their top-down controls of herbivores, and (2) bottom-up trophic cascades (i.e., indirect effect through herbivores to carnivores). First, predators, such as parasitoid wasps included in the carnivore group, are well-known to be attracted by VOC emission from plants (De Moraes et al. 1998). Concentration and composition of VOC emission usually vary among plant genotypes (Loughrin et al. 1995; Gouinguené et al. 2001; Wason and Hunter 2014). In fact, the most constant positive correlation between genetic distance and dissimilarity of the carnivore group was found throughout the two years (Fig. 2-4d). Moreover, significant relationships from the carnivore group to two herbivore groups were detected in both years (Fig. 2-5, 7). The attracted predators could then affect herbivorous insects. Second, bottom-up trophic cascades usually emerge from differences in host plant traits to predator communities mediated through abundance and composition of herbivores (Utsumi and Ohgushi 2009). Moreira and Mooney (2013) found that genetic diversity of a host plant species indirectly increased the abundance of a predator population by increasing the abundance of an aphid population. Thus, the robust relationship between genetics in the foundation trees and associated arthropod communities was supported by both direct effects of plant traits on predators and their top-down controls as well as bottom-up trophic cascades. It would result in constant genetic effects on predators and temporally variable effects on herbivore communities.

In conclusion, this study clearly demonstrated the important effects of genetic variation within a foundation species on associated community structure in a natural forest ecosystem. These results obtained by genome-wide analysis provide great insights into understanding how community structure is shaped in a complex ecosystem based on natural genetic variation generated through a local evolutionary history. As a next step, future studies should unravel a mechanistic connection between local evolutionary history and assembly of emerging associated communities in a natural complex ecosystem.

Tables

Table 2-1. Locations of study sites. We surveyed riparian forests in four river branches of Shumarinai lake, Hokkaido, Japan.

Site	*Latitude*	*Longitude*
1	44° 25’ 36.99”	142° 12’ 55.84”
2	44° 23’ 41.05”	142° 13’ 22.42”
3	44° 22’ 27.98”	142° 13’ 08.60”
4	44° 18’ 55.58”	142° 09’ 16.68”
5	44° 25’ 05.44”	142° 12’ 12.87”
6	44° 23’ 55.80”	142° 11’ 59.89”
7	44° 22’ 59.41”	142° 11’ 37.53”
8	44° 22’ 38.96”	142° 10’ 56.04”
9	44° 11’ 25.49”	142° 08’ 11.76”
10	44° 07’ 41.22”	142° 10’ 01.29”
11	44° 23’ 30.98”	142° 08’ 01.88”
12	44° 22’ 52.04”	142° 08’ 42.65”

Table 2-2. Mantel tests and partial mantel tests between community dissimilarity and measured factors. We tested correlation between pair-wise Set1 (community dissimilarity of arthropod) and Set2 (GD: genetic distance; LD: leaf trait distance; PCD: Phylogenic community dissimilarity of trees; SD: spatial distance; RD: distance along the river; TD: temporal difference of surveyed date) using mantel tests. Correlation between community dissimilarity of arthropods and genetic distance of alders was tested correcting LD, PCD, SD, RD, or TD with partial Mantel tests. Bold values indicate significant correlations at the level of 0.05. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

				Morisita-Horn		**Bray-Curtis**	
				2014	2015	2014	2015
Set1	Set2	Corrected for		*r*			
Dissimilarity of arthropod community	GD			**0.742*** **	**0.473***	**0.718****	**0.496***
	LD			**0.509****	**0.345***	**0.452***	0.338
	PCD			0.178	0.316	0.224	**0.399***
	SD			-0.006	-0.037	0.035	0.051
	RD			0.232	-0.044	0.177	0.042
	TD			0.146	**0.189***	0.193	0.168
	GD	-	LD	**0.747*** **	**0.455***	**0.726****	**0.480***
	GD	-	PCD	**0.735*** **	**0.451***	**0.708*** **	**0.477***
	GD	-	SD	**0.753*** **	**0.474***	**0.735*** **	**0.514***
	GD	-	RD	**0.742*** **	**0.479***	**0.717*** **	**0.495***
	GD	-	TD	**0.741*** **	**0.470***	**0.716****	**0.493***

***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$

Table 2-3. Composition of each functional group. The chewer group is constructed by 31 species. The sap-feeder group is constructed by 29 species. The spider group is constructed by 10 species. The carnivore group is constructed by 22 species. The other group is constructed by 23 species.

Functional group	*Families*
Chewer 2014: 278 individuals 2015: 246 individuals	Curculionidae, Chrysomelidae, Scarabaeidae, Lagriidae, Elateridae, Cerambycidae, Lymantriidae, Attelabidae, Tenthredinidae
Sap-feeder 2014: 594 individuals 2015: 1945 individuals	Miridae, Derbidae, Aphididae, Iassidae, Cicadellidae, Coccidae, Aphrophoridae, Idioceridae, Pentatomidae, Membracidae, Psyllidae, Drabescidae, Deltocephalidae, Lygaeidae, Acanthosomidae, Evacanthidae, Dictyopharidae
Spider 2014: 850 individuals 2015: 516 individuals	Thomisidae, Theridiidae, Nesticidae, Salticidae, Araneidae, Tetragnathidae, Clubionidae
Carnivore 2014: 291 individuals 2015: 237 individuals	Pentatomidae , Sphecidae, Formicidae, Ichneumonidae, Blaconidae, Harpalidae, Reduviidae, Cantharidae, Tenthredinidae, Pompilidae, Coccinellidae
Other 2014: 291 individuals 2015: 108 individuals	Scarabaeidae, Forficulidae, Chrysopidae, Alleculidae, Cantharidae, Mordellidae, Elateridae, Lycidae, Psocidae, Osmylidae, Rhizophagidae, Trixagidae, Lampyridae, Scolytidae

Table 2-4. Total number of tree species individuals surrounding alders. Each abbreviation means following species: B. er is *Betula ermanii*, S. sa is *Salix sachalinensis*, A. hi is *Alnus hirsuta*, S. mi is *Salix miyabeana*, F. ma is *Fraxinus mandshurica*, U. da is *Ulmus davidiana*, B. pl is *Betula platyphylla*, S. pe is *Salix pet-susu*, S. ba is *Salix bakko*, A. mo is *Acer mono*, S. in is *Salix integra*, P. am is *Phellodendron amurense*, and J. ai is *Juglans ailanthifolia*.

Species	*Families*	abundance
B. er	Betulaceae	2
S. sa	Salicaceae	129
A. hi	Betulaceae	224
S. mi	Salicaceae	43
F. ma	Oleaceae	9
U. da	Ulmaceae	16
B. pl	Betulaceae	28
S. pe	Salicaceae	2
S. ba	Salicaceae	4
A. mo	Sapindaceae	2
S. in	Salicaceae	10
P. am	Rutaceae	1
J. ai	Juglandaceae	2

Table 2-4. Mantel tests and partial Mantel tests between dissimilarity of each functional group and measured factors. I tested correlation between pair-wise Set1 (dissimilarity of arthropod group) and Set2 (GD: genetic distance between pairs of *A. hirsuta*; LD: leaf trait distance; PCD: phylogenic community dissimilarity of trees; SD: spatial distance; TD: temporal difference) using Mantel tests. Correlation between community dissimilarity and genetic distance was tested correcting LD, PCD, SD or TD with partial Mantel tests.

2014							
		Set1	**Chewer**	**Sucker**	**Spider**	**Carnivore**	**Other**
Set2	Corrected for				*r*		
GD			**0.372** *	-0.065	**0.347** *	**0.640** **	0.049
LD			0.032	-0.054	-0.055	0.250	-0.134
PCD			0.143	0.212	**0.295** *	0.294	0.046
SD			0.104	0.132	-0.076	0.104	0.036
TD			0.108	0.039	-0.005	0.130	0.037
GD	-	LD	**0.371** *	-0.057	**0.359** *	**0.630** **	0.070
GD	-	PCD	**0.357** *	-0.103	**0.317** *	**0.628** **	0.042
GD	-	SD	**0.398** **	-0.043	**0.340** *	**0.671** **	0.056
GD	-	TD	**0.367** *	-0.068	**0.348** *	**0.638** **	0.047
2015							
GD			-0.038	0.261	0.128	**0.401** *	0.278
LD			0.080	**0.387** *	-0.067	0.177	0.200
PCD			0.016	**0.442** **	-0.168	0.187	**0.387** *
SD			0.148	0.261	0.127	-0.040	0.176
TD			0.119	0.025	-0.015	0.105	-0.059
GD	-	LD	-0.050	0.224	0.139	**0.385** *	0.257
GD	-	PCD	-0.041	0.214	0.159	**0.382** *	0.237
GD	-	SD	-0.013	0.322	0.153	**0.400** *	0.318
GD	-	TD	-0.047	0.260	0.129	**0.397** *	0.284

**: $P < 0.01$, *: $P < 0.05$

Table 2-6. Mantel tests between leaf trait distance and measured factors. I tested correlation between pair-wise Set1 (leaf traits distance) and Set2 (GD: genetic distance between pairs of *A. hirsuta*; PCD: phylogenic community dissimilarity of trees; SD: spatial distance; TD: temporal difference) using Mantel tests.

		2014	**2015**
Set1	Set2	*r*	
Leaf traits distance	GD	**0.592 ****	0.147
	PCD	-0.055	0.169
	SD	-0.230	0.144
	TD	0.112	-0.063

**: $P < 0.01$

Table 2-7. Mantel and partial Mantel tests between the year-to-year changes in the community dissimilarity and the predictors. I tested correlation between pair-wise Set1 (the vector difference of year-to-year changes in the community dissimilarity) and Set2 (GD: genetic distance between pairs of *A. hirsuta*; LD: leaf trait distance; PCD: phylogenic community dissimilarity of trees; SD: spatial distance; TD: temporal difference) using Mantel tests. The correlations between the year-to-year changes and the genetic distance was tested correcting LD, PCD, SD or TD with partial Mantel tests.

Set 1	Set2	Corrected for	*r*
The vector difference of year-to-year changes	GD		**0.669 ***
	LD		**0.635 ***
	PCD		0.318
	SD		-0.260
	TD		0.107
	GD	- LD	**0.4973 ***
	GD	- PCD	**0.6598 ***
	GD	- SD	**0.6562 ***
	GD	- TD	**0.6646 ***

*: $P < 0.05$

Table 2-8. Path coefficients of direct and indirect effects from gene or traits to the abundance of functional groups of SEM in 2014 (Fig. 2-7). The values indicate standardized path coefficients. Bold values indicate significant relationships and the significance is represented by the number of asterisks (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). The values in parentheses indicate indirect path coefficients except traits.

		CHEWER	SAP	CARNIVORE	SPIDER
Bottom-up model					
GENE1	Direct	-	**0.588*** **	-	**-1.258*** **
	Indirect	- (-)	- (-)	-0.068 (0.382)	1.050 (1.069)
GENE2	Direct	**-0.341*** **	-	-	**-1.030** **
	Indirect	- (-)	- (-)	-0.641 (-)	1.094 (0.442)
TRAIT1	Direct	-	-	**0.812*** **	**-0.825***
	Indirect	-	-	-	-
TRAIT2	Direct	-	-	0.257	0.414
	Indirect	-	-	-	-
TRAIT3	Direct	**-0.331** **	-	-	**-0.351***
	Indirect	-	-	-	0.429
Top-down model					
GENE1	Direct	**-0.437***	0.303	-	-
	Indirect	0.117 (-)	-0.169 (-)	-0.220 (-)	-0.416 (-)
GENE2	Direct	**-0.551*** **	-	-	-
	Indirect	0.279 (-)	0.063 (-)	-0.526 (-)	- (-)
TRAIT1	Direct	**-0.876** **	**-0.666** **	**0.666** **	-
	Indirect	0.523	0.586	-	-
TRAIT2	Direct	-	**-0.309***	-	**0.589***
	Indirect	-	0.273	-	-
TRAIT2	Direct	**-0.299*** **	-	-	-
	Indirect	-	-	-	-

Table 2-9. Path coefficients of direct and indirect effects from gene or traits to the abundance of functional groups of SEM in 2015 (Fig. 2-5). The values indicate standardized path coefficients. Bold values indicate significant relationships and the significance is represented by the number of asterisks (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). The values in parentheses indicate indirect path coefficients except traits.

		CHEWER	SAP	CARNIVORE	SPIDER
Bottom-up model					
GENE1	Direct	**0.705*** **	**0.454*** **	**-0.898** **	-
	Indirect	- (-)	0.230 (-)	0.905 (0.600)	-0.125 (-0.481)
GENE2	Direct	**-0.475*** **	**0.211***	**-0.573** **	**0.513** **
	Indirect	- (-)	- (-)	0.279 (0.279)	-0.223 (-0.223)
TRAIT1	Direct	-0.246	**0.415*** **	**-0.883** **	-
	Indirect	-	-	0.549	-0.440
TRAIT2	Direct	-	**-0.444*** **	-	**-1.157*** **
	Indirect	-	-	-0.587	0.470
Top-down model					
GENE1	Direct	**0.771*** **	**0.500*** **	-0.424	-
	Indirect	0.025 (-0.138)	-0.022 (-0.120)	0.347 (-)	0.260 (-)
GENE2	Direct	**-0.464*** **	**0.253*** -**	-	-
	Indirect	- (-)	- (-)	- (-)	- (-)
TRAIT1	Direct	-	**0.533*** **	**-0.471** *	-
	Indirect	-0.154	-0.134	-	-
TRAIT2	Direct	-	**-0.377*** **	**-0.669** *	**-0.507** *
	Indirect	-0.218	-0.190	-	-

FIGURE LEGENDS

Fig. 2-1. Correlations of Morisita-Horn dissimilarity with genetic distance of alders. Plots indicate genetic distance (x axis) and community dissimilarity (y axis) between pairs of each site in 2014 and 2015. The *r* and *P* values correspond to the results from Mantel tests. A solid line and a dash line indicate correlation in 2014 (representing by circles) and 2015 (representing by crosses), respectively.

Fig. 2-2. Proportion of unique and shared deviance explained on dissimilarity of arthropod communities. Proportions are given for communities in 2014 and 2015, and represent deviance that is explained by following predictors. GD: genetic distance (black); OTHER: measured predictors including phylogenetic community dissimilarity of trees, spatial distance between pairs of alders and temporal distance (gray); SHARED: both predictors (white).

Fig. 2-3. Correlation between year-to-year changes and genetic distance. X axis indicates genetic distance (x axis). Y axis indicates difference of the vector of year-to-year changes in community dissimilarity between pairs of sites. The *r* and *P* values correspond to the results from Mantel tests.

Fig. 2-4. Correlation between genetic distance of *A. hirsuta* and dissimilarity of each functional group in 2014 or 2015. Each figure indicates correlation between dissimilarity of the chewer group (a), the sap-feeder group (b), the spider group (c), the carnivore group (d) or the other group (e) and genetic distance using Mantel tests. The *r* and *P* values corresponded to the results from Mantel tests. A solid line and a dash line in each figure indicate correlation in 2014 (circles) and 2015 (crosses), respectively.

Fig. 2-5. Structural equation models for interactions between genetic variation in alders and arthropod communities through the bottom-up or the top-down effects in 2015. **a, b** My proposed hypothetical models considering the bottom-up and the top-down effects, respectively. **c, d** The best structural models through the bottom-up and the top-down effects, respectively. Solid arrows indicate positive effects. Dash arrows indicate negative effects. Bold arrows indicate significant effects (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Numerical values on each arrow indicate the standardized coefficients of each path. TRAIT1 and 2 represent the first and second PCoA axes of leaf trait dissimilarity, respectively. GENE1 and 2 indicate the first and second PCoA axes of genetic distance of alders, respectively.

Fig.2-6. Correlogram of community similarity of arthropods in 2014 (a) and 2015 (b), and genetic similarity of alders (c) vs. spatial distance (km). Black points indicate significant correlation. These correlograms were performed with 999 permutations, using vegan package.

Fig. 2-7. Structural equation models for interactions between genetic variation in alders and arthropod communities through the bottom-up or the top-down effects in 2014. **a, b** Our proposed full models considering the bottom-up and the top-down effects, respectively. **c, d** Best structural models through the bottom-up and the top-down effects, respectively. Solid arrows indicate positive effects. Dash arrows indicate negative effects.

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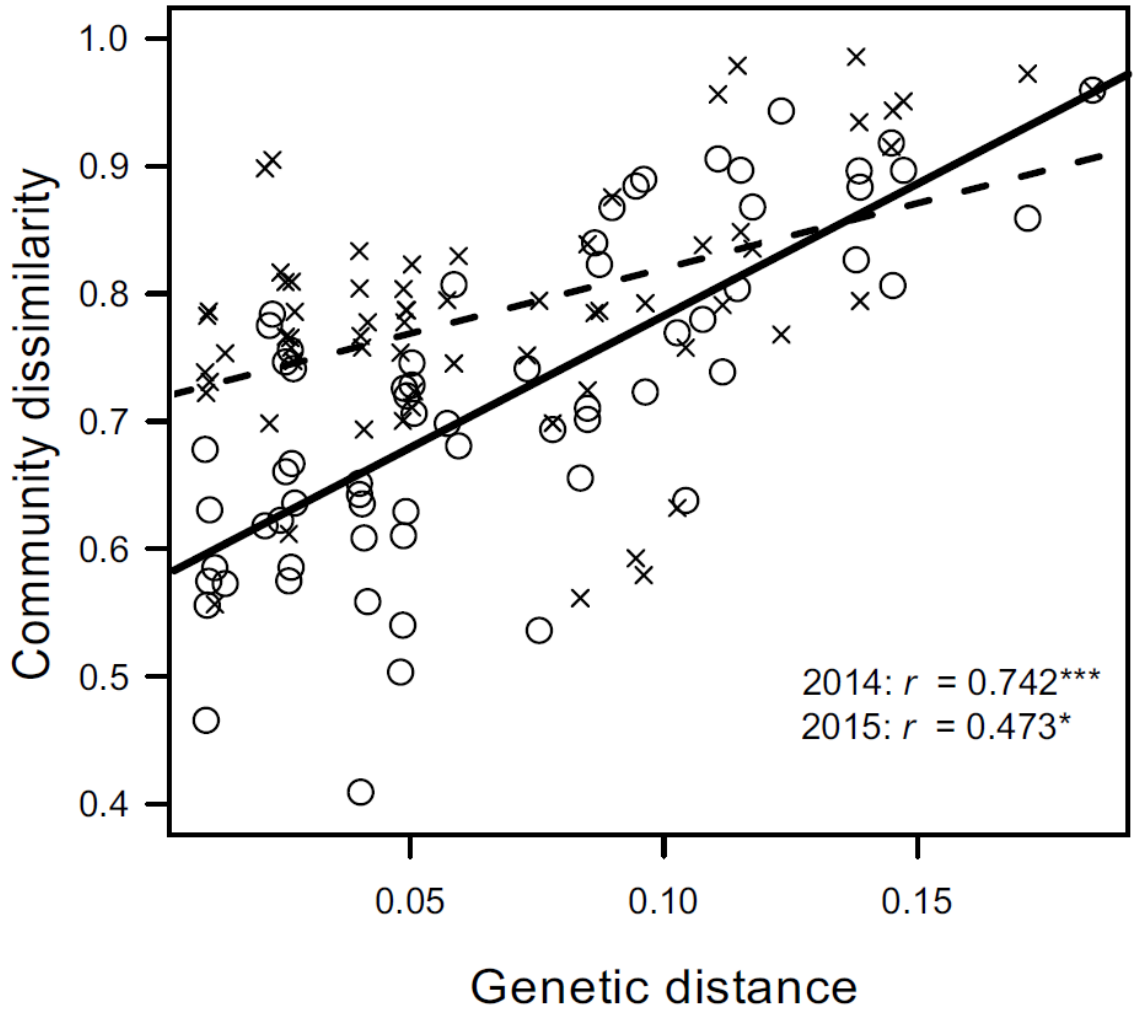

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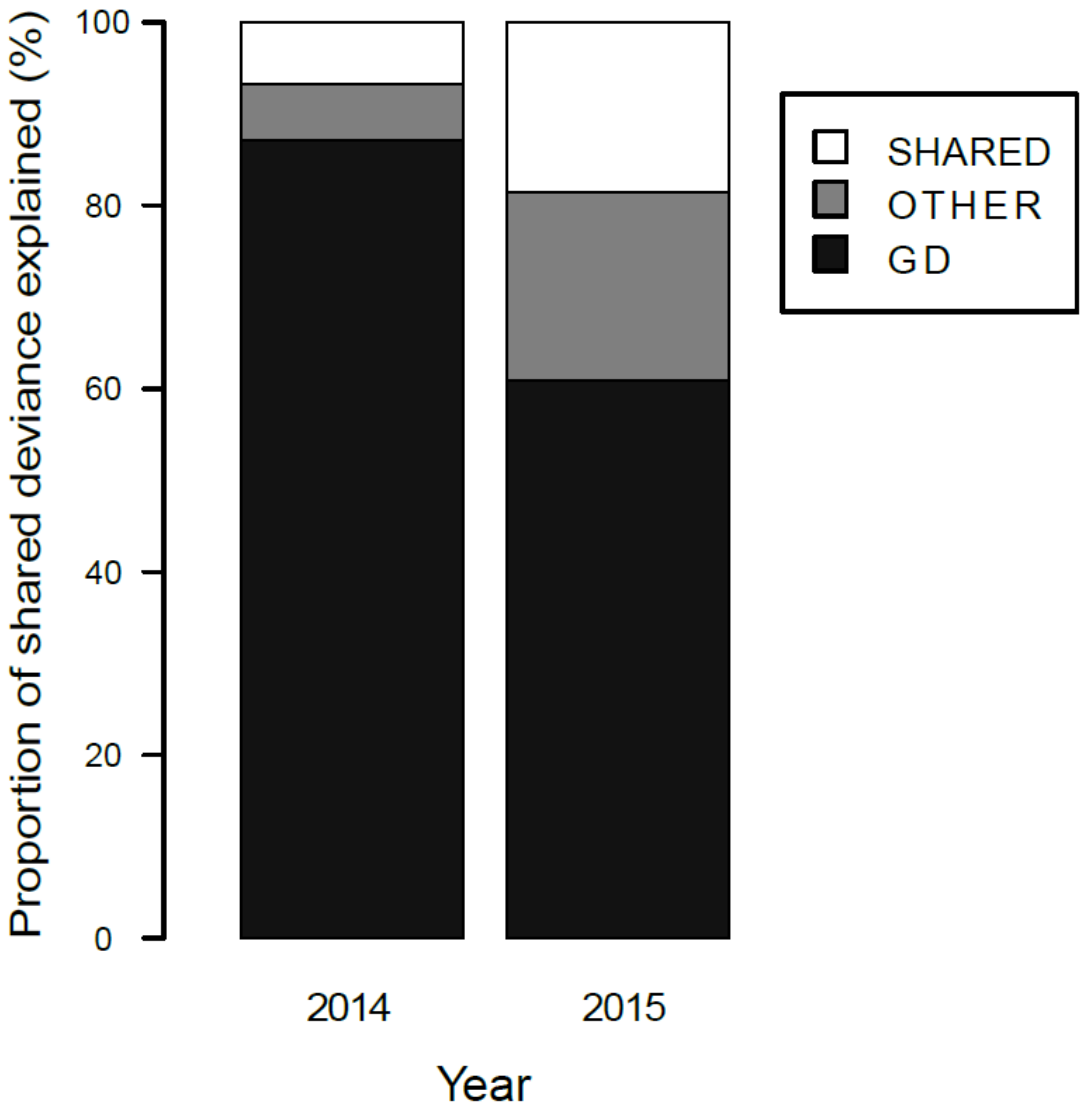

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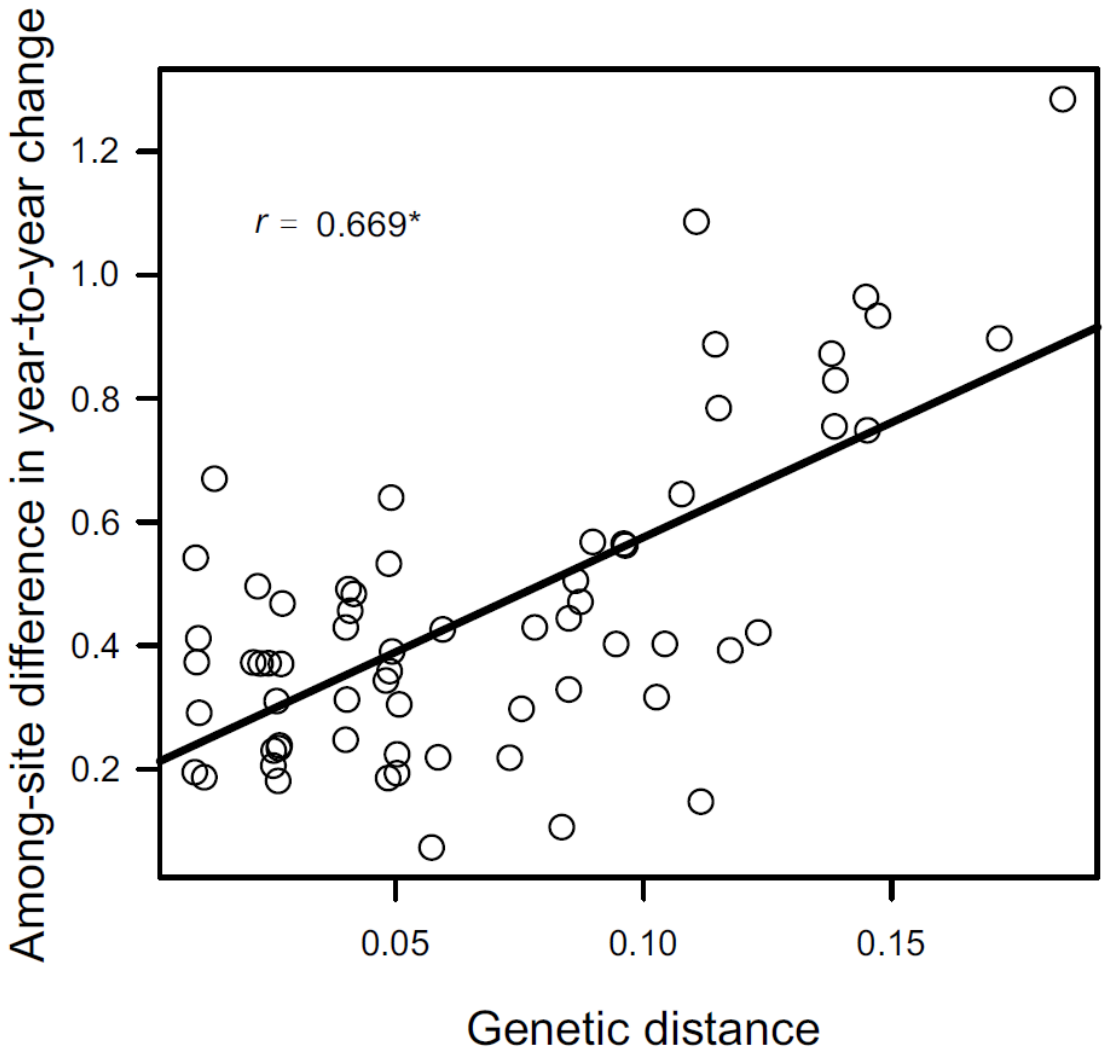

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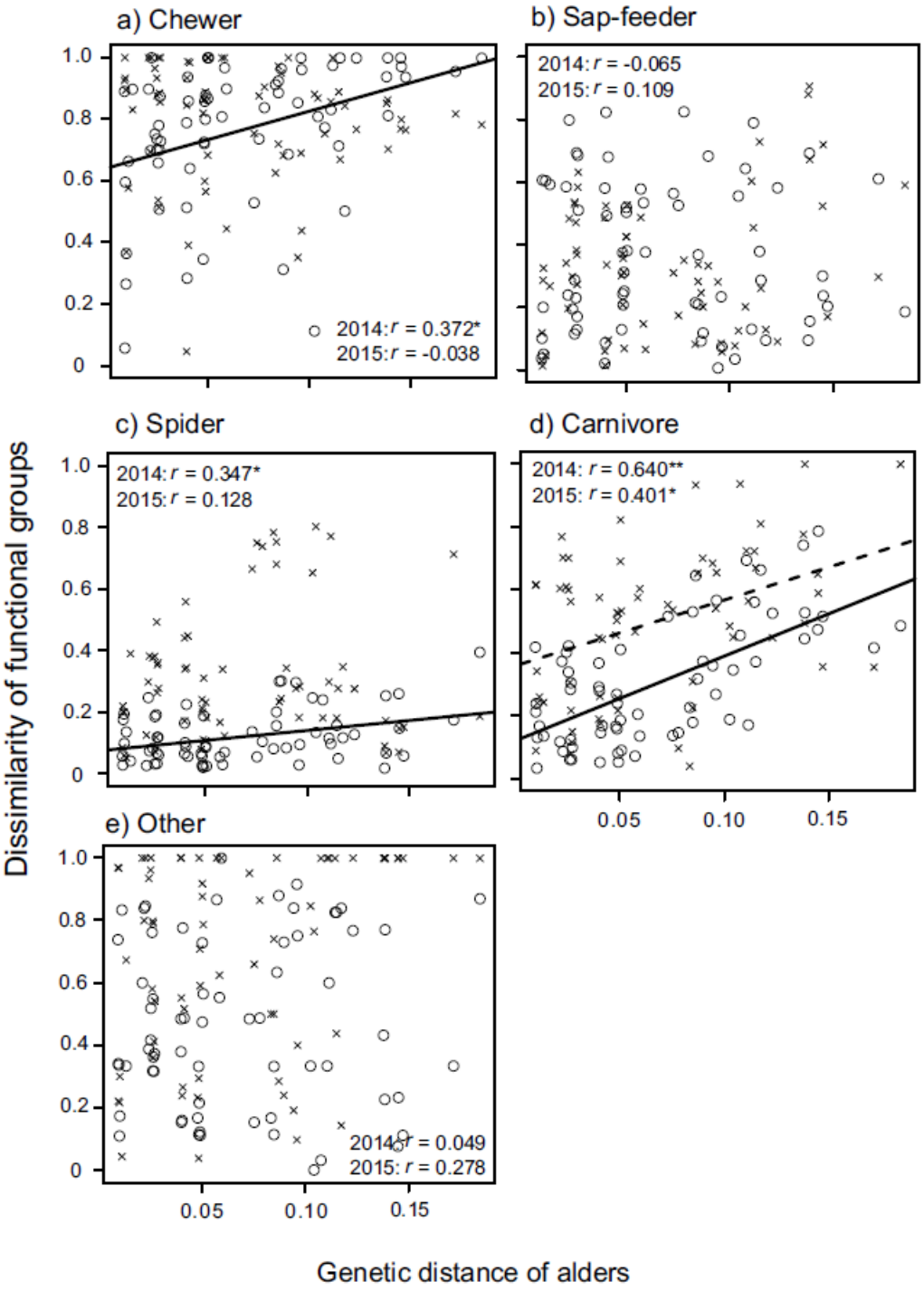

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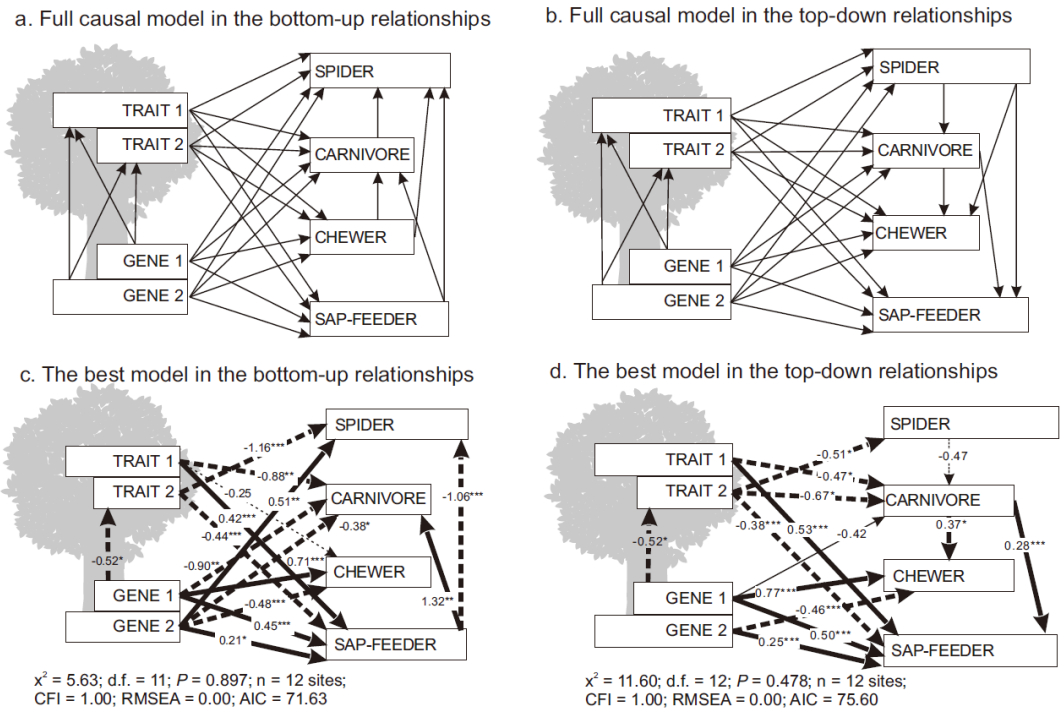

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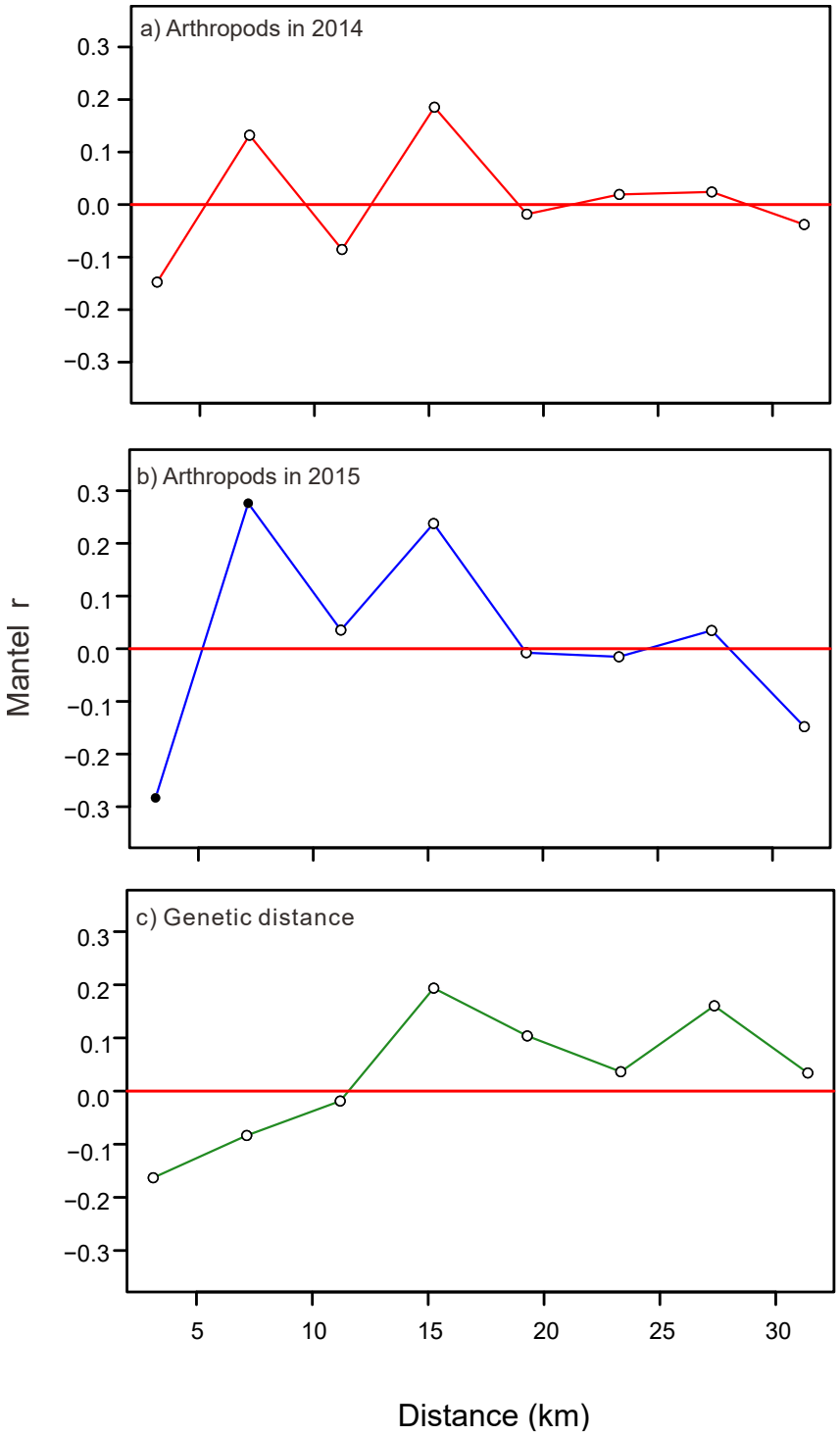

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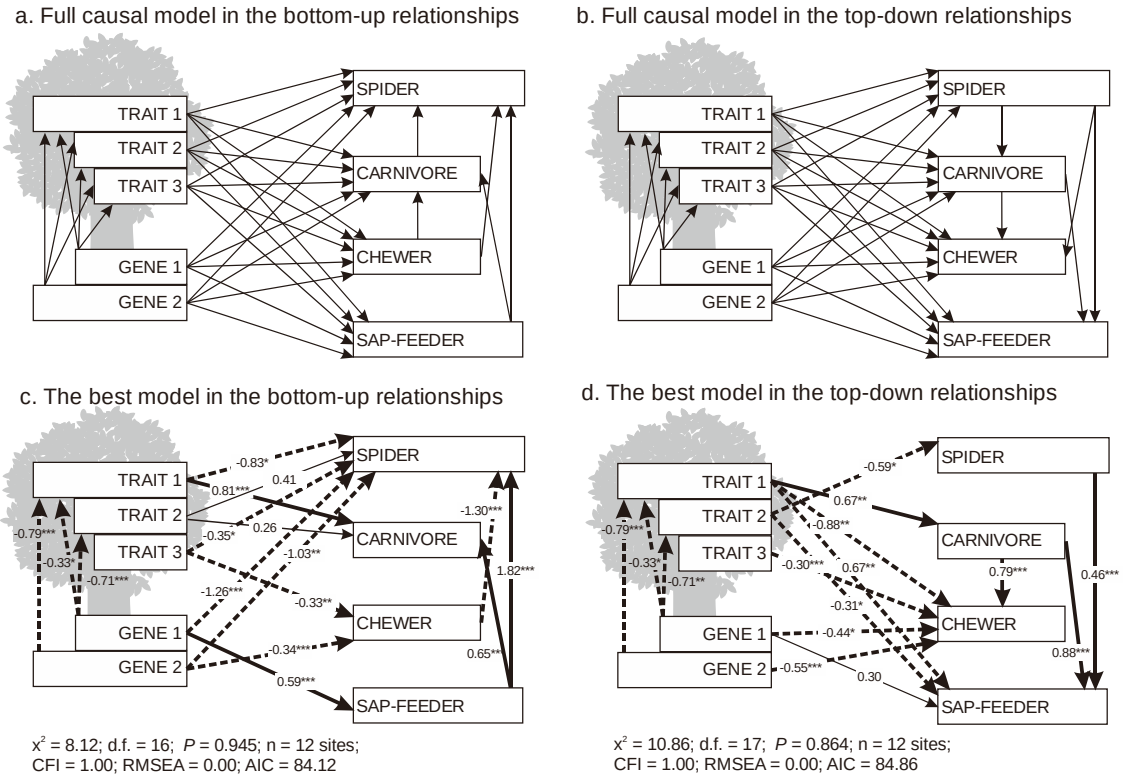

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Chapter 3. Spatial heterogeneity in genetic diversity and composition of bacterial symbionts in a single host species population

Introduction

Most terrestrial plants interact with microsymbionts in the rhizosphere. Root nodule symbiosis between plants and nitrogen-fixing bacteria has a significant impact on the nitrogen cycle in terrestrial ecosystems. In both evolutionary and applied biology, genetic variation in rhizobial mutualism has attracted considerable attention (Barrett et al. 2012; Miller and Sirois 1982; Robinson et al. 2000). Typically, experimental inoculation studies using different strains of bacterial symbionts have reported different effects of the mutualistic interactions, such as growth, nitrogen contents, and leaf size of the host plants, as well as nitrogen-fixation activity of the bacteria (Dillon and Baker 1982; Hooker and Wheeler 1987; Prat 1989; Sellstedt et al. 1986). In other words, it has been widely acknowledged that intraspecific variation in mutualistic nitrogen-fixing bacteria greatly affects host plant performance in terms of growth, survival, reproduction, and defense (Ballhorn et al. 2017; Barrett et al. 2012; Dean et al. 2014; Miller and Sirois 1982; Pahua et al. 2018; Prat 1989; Robinson et al. 2000), which in turn influences nitrogen-cycling processes. Thus, the knowledge of genetic variation in rhizobial mutualism is essential to understand not only the establishment and maintenance of a symbiosis but also wider ecosystem processes.

Nitrogen-fixing *Frankia* bacteria form nodules on the roots of actinorhizal plants. Many studies have focused on legume–rhizobia symbioses due to the agricultural importance, while interactions between actinorhizal plants and *Frankia* have been poorly examined. Whereas many legume plants are herbaceous, most actinorhizal plants are woody (Wheeler *et al*., 2008), and actinorhizal symbiosis is a major contributor to the global nitrogen budget in forest ecosystems, playing a dominant role in forest succession, especially in temperate and polar ecosystems (Kucho et al. 2010; Lawrence et al. 1967).

Therefore, the genetics of actinorhizal plants–*Frankia* bacteria mutualism may be more important than legume–rhizobia interactions on ecosystem processes in non-agricultural fields. Unraveling spatial structure of genetic diversity of mutualistic bacteria in non-agricultural field is likely to be important toward an understanding of nitrogen cycling, associated community dynamics, and coevolutionary dynamics in nodulation symbiosis. Nevertheless, there is only a small body of literature on the genetic diversity of *Frankia* in natural ecosystems (Anderson et al. 2009; Ben Tekaya et al. 2018; Benson and Hanna 1983; Clawson et al. 1998; Clawson et al. 1999; Huguet et al. 2001; Kennedy et al. 2010; Mishra et al. 2015; Pozzi et al. 2018a; Pozzi et al. 2015; Pozzi et al. 2018b; Ridgway et al. 2004; Roy et al. 2017; Simonet et al. 1994; Simonet et al. 1989; Vanden Heuvel et al. 2004; Wilcox and Cowan 2016).

Researchers recently have begun to reveal *Frankia* genetic diversity in wide geographic ranges. For example, Nouioui et al. (2014) investigated the genetic structure of *Frankia* on a global scale. The maximum distance of their study sites was approximately 19,000 km. Kennedy et al. (2010) and Wilcox and Cowan (2016) surveyed in regions where the maximum distances were 336.8 km and 165.0 km respectively. Some previous studies have investigated genetic diversity of *Frankia* on small spatial scales and/or from a single host species (Benson and Hanna, 1983; Clawson et al. 1999; Khan et al. 2007; Mishra et al. 2015; Pokharel et al. 2011; Pozzi et al., 2015; Simonet et al. 1994; Simonet et al. 1989). However, the above studies assessed genetic diversity of *Frankia* with small sample size per study sites. Therefore, distribution of *Frankia* genetic diversity within a small spatial scale has been overlooked.

The most important reason why the knowledge of genetic diversity of *Frankia* is still limited on a small spatial scale may be assumption that genetic diversity of mutualistic

partners is low on a local scale and in a single host species. The traditional mutualistic theory has suggested that the genetic diversity of mutualistic partners is constrained by hosts and could be decreased by the hosts' stabilizing mechanisms, such as partner choice and sanction (Archetti et al. 2011; Heath and Stinchcombe 2014).

It should also be noted that most previous studies compared genetic variation in *Frankia* among host plant species. This is because the focus has mainly been on the symbiotic host specificity in this mutualism (i.e., differences in the infectivity of rhizobial symbionts among host plant species; Baker 1987; Jiabin et al. 1985; Mirza et al. 2009). In natural ecosystems, different host species commonly associate with phylogenetically different *Frankia* strains (Du and Baker 1992; Normand et al. 1996). For this reason, most previous studies have compared genetic variation of *Frankia* among multiple host species to an understanding of coevolutionary history and effects of actinorhizal mutualism.

However, the knowledge of genetic diversity of *Frankia* on a small spatial scale (e.g., seed dispersal range: many seeds of *Alnus* individuals dispersed within *c.* 140 m along a river (Cunnings et al. 2016)) should be required to understand outcomes of considerable variation in current actinorhizal ecological interactions, such as the effectiveness of the bacteria in growth and survival of the host plants. This is because effects of rhizobial mutualism often depend not only on genetic variation of mutualistic bacteria but also on intraspecific variation of host species (Caldwell 1966; Hayashi et al. 2012; Heath and Tiffin 2007; Yamakawa et al. 2003). For example, nodulation rates of *Frankia* strains could also differ among intraspecific host individuals (Hahn et al. 1988). In fact, large genetic variation in a host plant population, including actinorhizal and legume species, is also ubiquitous in a natural forest stand (Ager et al. 1993; Kagiya et al. 2018; King and Ferris 1998; Wickneswari and Norwati 1993). My previous study revealed large genetic

variation in a single *Alnus* species in a continuous natural forest (20 km × 70 km; Kagiya et al., 2018). Leaf traits, such as C:N ratio, leaf mass per area (LMA), and herbivory rate, varied with the genetic variation and localities with the forest. Therefore, I should pay attention to genetic diversity of rhizobial bacteria and its spatial heterogeneity on a small spatial scale, which may be crucial to determining outcomes of ecological interaction between rhizobial bacteria and actinorhizal host plants under natural ecosystem conditions.

In this study, my goal is to elucidate spatial structure in genetic diversity and composition of mutualistic bacteria even in a local population of single host species (a single-host–population scale). Specifically, I sought to answer the following questions: (1) how diverse genetically is *Frankia* bacteria within and across local sites; (2) do the genetic compositions of *Frankia* bacteria differ among local sites; and (3) what is the spatial genetic structure of *Frankia* in a natural forest. For the purposes of the study, I focused on the *A. hirsuta–Frankia* symbiosis in a natural forest in northern Hokkaido, Japan. Actinorhizal populations in this forest region are dominated by a single *Alnus* species, *A. hirsuta*. The genetic variation of *A. hirsuta* within the forest has been determined by a genome-wide analysis (Kagiya et al. 2018). I continuously investigated the genetic diversity of *Frankia* bacteria in the *Alnus* populations at intervals of *c.* 100 m (the maximum distance between host populations is 43.476 km; 213 seedlings in total).

Materials and Methods

Host species and nitrogen-fixing bacteria

Alnus hirsuta (Betulaceae; *Alnus incana* ssp. *hirsuta* Spach; Chen and Li 2004; Ren et al. 2010) is a deciduous broadleaf tree and early successional species. It is widely distributed

in temperate riparian forests of Japan, northeastern China, Korea, and Russia. *Alnus* trees have the following characteristics as foundation species in a riparian forest ecosystem (Ellison et al. 2005; 2010): (1) they are a dominant species in early succession forests, (2) they support diverse arthropod species (Kagiya et al. 2018; Nyeko et al. 2002), and (3) they are actinorhizal species able to establish symbiosis with nitrogen-fixing actinobacteria, *Frankia* sp. (Frankiaceae) forming nodules in their roots, which seem to greatly affect ecosystem processes such as nutrient cycling. *Frankia* bacteria have the ability to convert atmospheric nitrogen into ammonia, and are free-living soil microbes but some are obligate symbionts (Benson and Dawson 2007).

Root nodule sampling

Our study sites are located in and around the Uryu Experimental Forest (44° 030–290N, 142° 010–200E) of Hokkaido University in northern Hokkaido, Japan. This experimental forest is a continuous mixed conifer–broadleaf forest of *c.* 25000 ha. One nodule was collected from each of the roots of 213 *A. hirsuta* seedlings (DBH: < 2 cm) from five riparian areas of the forest (BT, DRE, DRW, SE, and UT; the maximum distance between my areas was 43.5 km; Fig. 3-1) because the host trees are mainly found along rivers, and streams are considered one of the primary dispersal pathways of *Frankia* bacteria (Arveby and Huss-Danell 1988; Huss-Danell et al. 1997). Seedlings from which I collected root nodules were selected from 17 sites from the riparian areas. Sampling root nodules from host seedlings would allow us to collect samples continuously from a whole forest and to estimate genetic diversity and composition of *Frankia* which interact with a single host plant population at present. Sampling was conducted from June to September 2016. The

distance between sampling points in each site was more than 100 m. *Alnus hirsuta* is the only actinorhizal species in this forest.

Molecular Analyses

The collected nodules were surface-sterilized using 10% (v/v) Clorox bleach. DNA was extracted from root nodules using DNeasy Blood & Tissue Kit (Qiagen). The nodules were sliced using sterilized razor blades and crushed using sterilized homogenization sticks. The crushed lobes were heated to 37 °C for 30 min with a 25 µl Proteinase K. Polymerase chain reaction (PCR) was performed to amplify a 496-bp fragment of a *nif*D-K intergenic spacer region with the *Frankia*-specific primer pair (Anderson et al., 2013), *nif*D1310frGC (5'-CGC CAG ATG CAC TCC TGG GAC TAC T-3'), and *nif*KR331frGC (5'-CGG GCG AAG TGG CTG CGG AA-3'). I focused on intrageneric variation of *Frankia* based on the *nif*D-K intergenic spacer (IGS) region. This genetic marker is considered useful for resolution at the species level of *Frankia* because this genetic region is higher variable than ribosomal RNA (Anderson et al. 2009; Mishra et al. 2015). PCR amplification was performed as follows: 1 cycle at 95 °C for 2 min, followed by 35 cycles of 95 °C for 1 min and 64 °C for 5min, and a final step of 1 cycle at 72 °C for 5 min. The PCR products with expected size were cleaned using an ExoSAP master mix containing 0.5 µl Exonuclease I (TaKaRa), 0.5 µl Shrimp Alkaline Phosphatase (SAP; New England BioLabs), and 2.0 µl sterile deionized water. Incubation was conducted at 37 °C for 20 min and at 80 °C for 15 min. These products with *nif*KR331frGC were sequenced using an automated sequencer (3730xl DNA Analyzer, Applied Biosystems). DNA sequence chromatograms were manually checked using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com), and sequences were aligned using MEGA 7.0.21

(Kumar et al. 2016). Finally, nucleotides in obtained sequences were checked to remove sequences of low reliability. In total, 201 sequences were used for subsequent analyses.

Operational taxonomic unit (OTU) separation was performed to classify the 201 sequences at a 97.0%-threshold, using the CD-Hit program (Li and Godzik 2006). This threshold was decided based on the statistical method detailed in Põlme et al. (2014). These sequence data were deposited in the DNA Data Bank of Japan (DDBJ) with accession no. LC482655-LC482672. Phylogenetic trees were constructed using Maximum Likelihood (ML; bootstrap analyses with 1000 replications; Fig. 3-2) and Neighbor-Joining (NJ; bootstrap analyses with 10000 replications; Fig. 3-6) based on the Kimura 2-parameter evolutionary model (Kimura, 1980) with a discrete gamma distribution selected by evolutionary model selection procedure in MEGA 7.0.21 (Kumar et al. 2016). The phylogeny included the *nif*D-K locus of uncultured *Frankia* bacteria obtained from two *Alnus* species, *A. incana* (ssp. *tenuifolia* (Anderson et al. 2009) and ssp. rubra) and *A. viridis* (Anderson et al. 2009), and nine varieties of *Myrica rubra* (var. *biji*, var. *baimei*, var. *dongkui*, var. *muye*, var. *shuimei*, var. *wandao*, var. *wumei*, var. *yangliu*, var. *zaoda*; He et al. 2004), as well as a cultured ACN14a strain, whose host is *A. viridis* (Normand et al. 2007). Other *Frankia* sequences from different actinorhizal species, *Hippöphae salicifolia* (Mishra et al. 2015), three *Coriaria* species (*C. myrtifolia*, *C. japonica*, *C. arborea*; Nouioui et al. 2014), *Elaeagnus angustifolia*, *Datisca glomerate*, and *Casuarina equisetifolia* (Normand et al. 2007), were also included as outgroups. These sequences covered *Frankia nif*D-K sequences of almost actinorhizal hosts in GenBank. These sequences were obtained from GenBank. All positions with less than 95% site coverage were eliminated. Both ML and NJ phylogenetic trees were generated with MEGA 7.0.21 (Kumar et al. 2016). To describe relationships between *Frankia* OTUs

and areas, I also generated the ML phylogeny with 1000 bootstraps, excluding the sequences obtained from the database (Fig. 3-4).

To analyze the spatial genetic structure of *Frankia* in a natural habitat, analysis of molecular variance (AMOVA) was performed with 9,999 permutations, using GenAlex 6.5 (Peakall and Smouse 2012). The five riparian areas were divided into 2–5 sites each to analyze the genetic structure within the areas.

Analysis of *Frankia* diversity

To estimate spatial heterogeneity of the *Frankia* composition on a single-host–population scale, Bray-Curtis dissimilarity index was calculated. The total number of each *Frankia* OTU was used for the data set at the site- and area-level. The data set was standardized to unified scale [0:1], dividing by total number of each site/area, because sample sizes were different among sites/areas. The significance of *Frankia* composition dissimilarity among areas was analyzed using permutation MANOVA (PERMANOVA) with 9,999 permutations. To visually summarize the dissimilarity among sites, non-metric multidimensional scaling (NMDS) in a two-dimensional space was performed. All calculations in above were performed using the R package vegan 2.5-6 (Oksanen et al. 2019) with the R 3.6.1 software.

To consider spatial autocorrelation in the *Frankia* OTU compositions, I calculated spatial distance among sites and areas. Spatial distance was calculated based on location of each site/area with Great Circle distance method, using the R package sp 1.3-1(Pebesma et al. 2018). The location was calculated centroid location as the averaged latitude and longitude of each *A. hirsuta* seedling. Spatial autocorrelation of *Frankia*

compositions was analyzed by Mantel tests with 9,999 permutations. These tests were performed using the vegan package.

Results

Genetic diversity of *Frankia* on a single-host–population scale

To classify *Frankia* in the study forest, I used OTU methods based on the genetic similarity of the *nif*D-K loci. A total of 18 OTUs were identified at a 97.0% similarity based on the *nif*D-K loci of *Frankia* in the forest (Table 3-2, Fig. 3-2). This 97.0% threshold for OTUs is likely to be relevant to represent the phylogenetic relationship of *Frankia* strains based on the *nif*D-K ITS region because sequences of all samples were clearly clustered to each clade of single OTU (Fig. 3-7). The phylogenetic trees indicated that obtained OTUs widely spread in the almost range of *Alnus*-infective clade (Fig. 3-2, 3-6). Each of the three most common OTUs, OTU01, OTU02, and OTU03, was placed into phylogenetically different clades (Fig. 3-2, 3-6). Thus, my result demonstrated that genetically diversified strains co-occurred in the forest. In addition, both OTU06 and OTU07 were genetically close to OTU02, and both OTU04 and OTU05 were genetically close to OTU03. Some *Frankia* sequences in this study were phylogenetically close to bacteria from *A. incana* ssp. *tenuifolia* or *A. viridis*, which were belonging to different clades between the host species (Anderson et al. 2009; 2013). Additionally, it should be noted that seven of these OTUs (OTU01–OTU07) were obtained from multiple samples, while the rest (OTU08-OTU18) was rare singleton (Fig. 3-4, 3-7).

To illustrate differences in *Frankia* OTU diversity (i.e., the total number of OTUs in each site) among the sites with the standardization of sample size, I generated a rarefaction curve for each site (Fig. 3-3). OTU diversity was greater in BT, DRE, and

DRW areas than in SE and UT areas. While the rarefaction curves of SE and UT areas show the saturation of the total OTU number, those of BT, DRE and DRW areas indicated there was likely to be still undetected OTUs in each area.

Differences in *Frankia* composition

The three most abundant OTUs (i.e., OTU01, OTU02, and OTU03) were commonly found throughout the entire sampling areas (Fig. 3-4), indicating a sympatric coexistence of these haplotypes. However, other OTUs were localized to parts of different sampling areas. OTU04, OTU05, OTU06, and OTU07 were detected in seedlings from three riparian areas (BT, DRW, and DRE). These results indicated the spatial heterogeneity in *Frankia* compositions within a single-host–population scale.

This finding was supported by NMDS community ordination, in which *Frankia* compositions were diversified among areas within a single-host–population scale (Fig. 3-5). The stress of the NMDS was 0.070, indicating a good representation of the data in two-dimensional ordination plot. The significant differences in *Frankia* OTU compositions were detected (PERMANOVA; $P < 0.05$).

Spatial genetic structure of *Frankia*

The AMOVA indicated a significant genetic differentiation in *Frankia* genetic communities among riparian areas and sites (Table 3-1). In addition, no significant correlations were detected between the dissimilarity of *Frankia* compositions and spatial distance (at site level: $r = -0.1037$, $P = 0.6967$; at area level: $r = 0.4897$, $P = 0.1833$). This suggested that spatial structure of *Frankia* compositions was unlikely to be resulted from spatial autocorrelation.

Overall, the spatial heterogeneity in *Frankia* genetic variation was due to the differences in both OTU diversity and composition of *Frankia* strains.

Discussion

This study clearly demonstrated that multiple *Frankia* genotypes coexist even in an area within a single-host–population scale. *Frankia* genetic diversity in a single-host–population scale is comparable with previous studies that analyzed the *nif*D-K genetic region of *Alnus*-infection *Frankia* from multiple hosts and/or in regional scales. Rarefaction analysis showed that the existence of undetected OTUs is expected in some sites. Furthermore, differences in *Frankia* genetic diversity and composition were detected even within the small spatial scale (Fig. 3-4, 5). These results suggest that actinorhizal host individuals within the population can interact with different *Frankia* genotypes.

Frankia diversity and composition on small spatial scales

The maintenance mechanisms of sympatric coexistence of phylogenetically distant *Frankia* strains can contribute to understanding the spatial heterogeneity in *Frankia* diversity and composition on local scales. Three factors can explain why various genotypes of mutualistic partners coexist in the same habitat (Heath and Stinchcombe 2014): (1) a different selection on each genotype of the partners by genetic variation in hosts (i.e., G × G: genotype–by–genotype interactions), (2) genetic trade-offs between bacterial strains, and (3) different functions of multiple *Frankia* genotypes.

First, in natural ecosystems, it is likely that the genetic structure of *Frankia* is greatly restricted by the hosts' phylogeny (Anderson et al. 2009; Põlme et al. 2014; Pozzi et al. 2018). Benefits for host plants from root-nodulating symbionts also differ among different genotypes within a host species, as well as exhibiting interspecific variation (Caldwell 1966; Hayashi et al. 2012; Heath and Tiffin 2007; Yamakawa et al. 2003). Therefore, the sympatric coexistence of phylogenetically distant *Frankia* strains on a single-host–population scale may be at least partially explained by intraspecific variation of the host plant *A. hirsuta*. Determining the effects of intraspecific variation in a host species with G × G interactions in mutualism may be important to understanding how a stable coexistence of genetically diverse mutualistic partners is sustained on a small spatial scale.

Second, genetic trade-offs between different mutualism-related traits, if existent, can contribute to the maintenance of a stable coexistence of different *Frankia* strains. For example, if mutualistic efficiency is driven by a trade-off with the ability to compete, mutualistically efficient *Frankia* strains can sympatrically coexist with inefficient *Frankia* strains that have an advantage in intrageneric competition (Ferriere et al. 2002; Hoeksema and Kummel 2003). In actinorhizal symbiosis, *Alnus* trees often interact with different phenotypes of *Frankia* bacteria, including spore-positive strains hosting abundant sporangia inside plant cells, and sporangia-free, spore-negative strains (Pozzi et al., 2015; Torrey, 1987). Infectivity and nitrogen-fixing activity might be negatively associated between the spore-positive/negative strains (Markham, 2008; Pozzi et al., 2015).

Third, different functions of *Frankia* genotypes may contribute to the maintenance of their genetic variation within the same location. Nitrogen resources from rhizobial

symbionts increases not only the growth of the host plants, but also their resistance to herbivores (Ballhorn et al. 2017; Dean et al. 2014; Thamer et al. 2011). In addition, nitrogen fixed by associated rhizobacteria, including *Frankia*, can be stored in nodules and transported to aerial parts as these specific forms, such as amides and ureides (Berry et al. 2011). If the genetic variation of *Frankia* strains is responsible not only for the nitrogen supply but also the different forms of nitrogen, multiple functions of genetically diverse *Frankia* may complementally improve the overall host plant fitness in a complex ecosystem.

Thus, these three interpretations which are not mutually exclusive could complementarily contribute to explanation of the mosaic-like, spatial genetic structure of *Frankia*. In future studies, phenotypes and functions of different OTUs detected in this study should be investigated.

Spatial structure in local genetic communities of *Frankia*

The results also revealed a complex, mosaic-like, genetic structures of *Frankia* on a single-host–population scale (spatial differentiation of *Frankia* OTU components; Fig. 3-3) that did not depend on geographic distance. The explanations mentioned in the above section can also contribute to understanding of the spatial mosaic-like patterns observed in *Frankia* genetic communities. The heterogeneous spatial structure of the interactions between genetically diverse hosts and rhizo-microorganisms can exert selective pressure resulting in the spatial differentiation of *Frankia* communities. In fact, I detected a genetic differentiation of the alder host not only among the studied riparian areas but also within each area (Kagiya et al. 2018), as well as the spatial genetic structure of *Frankia*. However, a part of patterns in genetic structure of *Frankia* were inconsistent with the pattern of host

genetic structure. For example, *Frankia* compositions were different between BT and SE area (Fig. 3-5), while *A. hirsuta* populations were closely related. BT area is completely covered with natural forest stands but SE area is close to agriculture field and its riverside landscape is artificially modified. The differences in abiotic and biotic environments could affects the selection outcomes (e.g., G × G × E: genotype–by–genotype–by–environment interactions), which may contribute the observed mosaic-like structures of the *Frankia* communities. Future studies should pay attentions to associations between genetics of host individuals and mutualistic symbionts in a local scale.

Furthermore, the dispersion processes of *Frankia* may play a key role in generating these spatial patterns. The significant genetic differentiation of *Frankia* among riparian areas (Table 3-1) may be due to the dispersal of *Frankia* bacteria by the waterways (Arveby and Huss-Danell 1988; Huss-Danell et al. 1997). In addition, massive snow-melt in the study site (snow depth: > 200 cm) may also drive soil bacteria dispersion by transporting soil components along the complex river landscape. Previous studies suggested that herbivorous mammals (Chaia et al. 2012), birds (Paschke and Dawson 1993), and invertebrates such as earthworms (Reddell and Spain 1991) can also drive the dispersal of *Frankia*, carrying their propagules. *Frankia* propagules did not lose their activity to infect their hosts despite going through the digestive tracts of such animals (Burleigh and Dawson 1995; Chaia et al. 2012). Thus, the genetic mosaic-like structure can be, at least partially, the result of both biotic (e.g., deer, birds, and earthworms) and abiotic dispersion processes (snowmelt and waterways).

To my knowledge, this is the first study that demonstrated spatial heterogeneity in genetic diversity and composition of *Frankia* bacteria in a single-host–population scale. My findings suggest that actinorhizal host individuals may be exposed by different

Frankia strains within a population. The interactions with different genotypes of mutualistic bacteria widely influences phenotypes of host plants (Ballhorn et al. 2017; Barrett et al. 2012; Dean et al. 2014; Miller and Sirois 1982; Pahua et al. 2018; Prat 1989; Robinson et al. 2000). The variation in mutualistic partnerships on small spatial scales could increase the heterogeneity of ecosystem processes and/or associated community dynamics in forest ecosystems. Understanding genetic structure of nitrogen-fixing bacterial symbionts holds the key to elucidating these dynamics in forest ecosystems. Further research is required to shed more light on the mechanisms that create the spatial heterogeneity in genetic diversity and composition of actinorhizal symbionts on a local scale.

Tables

Table 3-1. Explanation of genetic structure by study areas/sites based on AMOVA.

Source	**df**	**Estimate**	***P***
Among areas	4	1.463	**0.015**
Among sites	12	2.521	**0.012**
Within sites	184	47.552	< **0.001**

Table 3-2. Numbers of OTUs obtained from each site.

Area	**BT**				**DRE**		**DRW**				**SE**				**UT**			
Site No.	1	2	3	4	1	2	1	2	3	4	1	2	3	4	1	2	3	Accession No.
OTU01	8	3	9	9	6	2	4	11	5	9	11	9	7	6	8	8	9	Ahi01 (LC482655)
OTU02	4	1	0	0	3	2	2	2	2	2	0	0	0	1	4	0	0	Ahi02 (LC482656)
OTU03	0	2	1	1	1	0	2	1	0	2	0	0	1	0	3	2	0	Ahi03 (LC482657)
OTU04	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	Ahi04 (LC482658)
OTU05	0	0	0	0	1	0	0	2	2	0	0	0	0	0	0	0	0	Ahi05 (LC482659)
OTU06	0	6	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	Ahi06 (LC482660)
OTU07	0	0	0	1	2	0	1	0	0	0	0	0	0	0	0	0	0	Ahi07 (LC482661)
OTU08	0	0	0	0	1	0	0	0	1	2	0	0	0	1	0	0	0	Ahi08 (LC482662)
OTU09	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Ahi09 (LC482663)
OTU10	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ahi10 (LC482664)
OTU11	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Ahi11 (LC482665)
OTU12	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Ahi12 (LC482666)
OTU13	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Ahi13 (LC482667)
OTU14	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Ahi14 (LC482668)
OTU15	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ahi15 (LC482669)
OTU16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Ahi16 (LC482670)
OTU17	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Ahi17 (LC482671)
OTU18	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Ahi18 (LC482672)
Total	13	14	11	11	16	4	10	17	15	15	12	10	9	9	15	10	10	

Table 3-3. Shannon diversity indices, evenness, richness and sample size of *Frankia* OTU compositions in each site and area and whole of the Uryu Experimental Forest.

	Region	Shannon	Evenness	Richness	Sample size
Site-level	BT1	0.8587	0.7817	3	13
	BT2	1.5367	0.8577	6	14
	BT3	0.6002	0.5463	3	11
	BT4	0.6002	0.5463	3	11
	DRE1	1.8080	0.8695	8	16
	DRE2	0.6931	1.0000	2	4
	DRW1	1.4708	0.9139	5	10
	DRW2	1.3157	0.7343	6	17
	DRW3	1.6792	0.8629	7	15
	DRW4	1.0776	0.7773	4	15
	SE1	0.2868	0.4138	2	12
	SE2	0.3251	0.4690	2	10
	SE3	0.6837	0.6224	3	9
	SE4	0.3488	0.5033	2	9
	UT1	1.0096	0.9190	3	15
	UT2	0.5004	0.7219	2	10
	UT3	0.3251	0.4690	2	10
Area-level	BT	1.3153	0.6759	7	49
	DRE	1.6923	0.8138	8	20
	DRW	1.6392	0.6836	11	57
	SE	0.5779	0.3226	6	40
	UT	0.8678	0.6260	4	35
Whole forest		1.4373	0.4973	18	201

Figure legends

Fig. 3-1. Map of the sampling points in the Uryu Experimental Forest of Hokkaido University, northern Hokkaido, Japan, where *A. hirsuta* seedlings root nodules were collected. Different colors indicate different areas: (a) overall map, (b–f) individual areas (b: UT; c: DRW; d: BT; e: SE; f: DRE). Marker shapes in magnified map areas (b-f) indicate sites within each area.

Fig. 3-2. Phylogenetic tree based on the *nif*D-K spacer region in *Frankia*. A maximum-likelihood phylogeny was generated based on 97 sequences of the *nif*D-K locus, obtained from three subspecies of *Alnus incana* (ssp. *hirsuta*: operational taxonomic units 'OTUs_' in this study; ssp. *rubra*: 'AR_'; ssp. *tenuifolia*: 'AT_'), *A. viridis* ('AV_'), nine varieties of *Myrica rubra* (var. *biji*: 'Mbj_'; var. *baimei*: 'Mbm_'; var. *dongkui*: 'Mdk_'; var. *muye*: 'Mmy_'; var. *shuimei*: 'Msm_'; var. *wandao*: 'Mwd_'; var. *wumei*: 'Mwy_'; var. *yangliu*: 'Myl_'; var. *zaoda*: 'Mdz_'), and the ACN14a strain as an *Alnus* infection clade. Other sequences obtained from *Hippöphae salicifolia* ('Hsli_'), three *Coriaria* species (*C. myrtifolia*: 'Cm_'; *C. japonica*: 'Cj_'; *C. arborea*: 'Ca_'), *Elaeagnus* ('EAN1pec'), *Datisca glomerate*, and *Casuarina equisetifolia* ('CcI3') were also included in the phylogeny as outgroups. These sequences were obtained from GenBank. The characters in parentheses indicate accession numbers on GenBank. Branch labels indicate significant bootstrap values. The tree is drawn to scale, with branch lengths measured according to the number of substitutions per site.

Fig. 3-3. *Frankia* diversity along sampling size in each site. Different colors indicate different areas

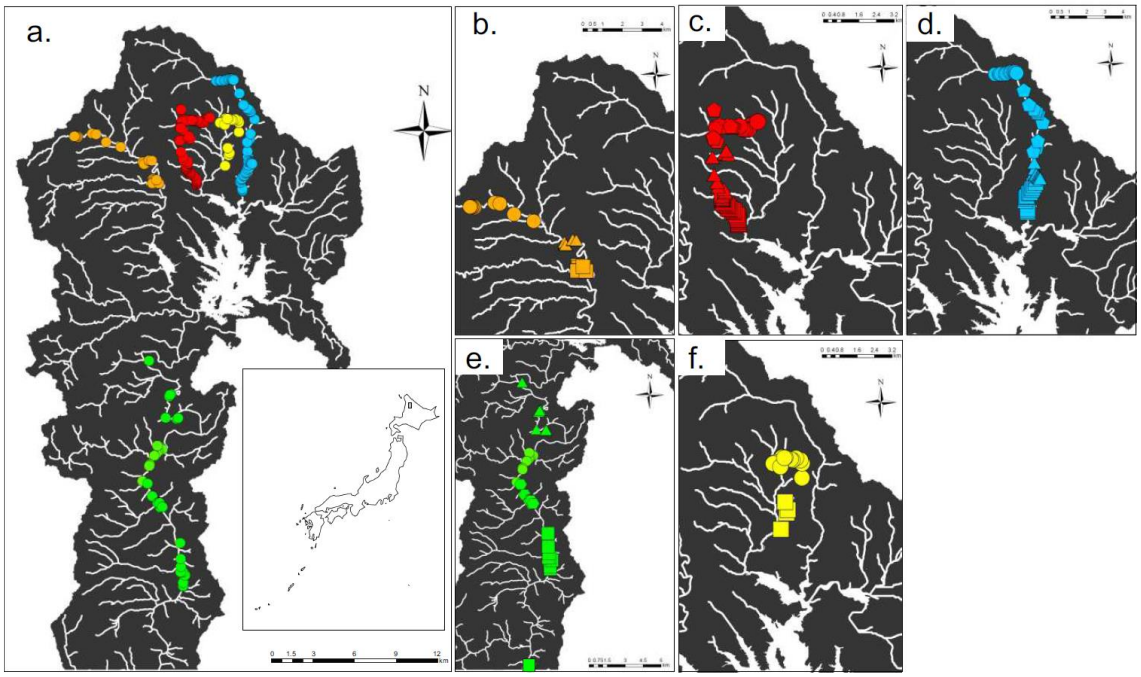
Fig. 3-4. Relationships between *Frankia* operational taxonomic units (OTUs) and areas. The phylogeny was generated using the maximum-likelihood method. Branch values indicate significant bootstrap values. Circles indicate the presence of each OTU in each site. Circle sizes represent numbers of OTUs in each site (see also Table 3-1).

Fig. 3-5. Non-metric multidimensional scaling (NMDS) of *Frankia* OTU compositions at the site-levels. Colors of points indicate areas. The numbers in points indicate ID of sites.

Fig. 3-6. Phylogenetic tree based on the *nif*D-K spacer region in *Frankia*. The neighbor-joining phylogeny was generated based on 97 sequences of the *nif*D-K locus, obtained from three subspecies of *Alnus incana* (ssp. *hirsuta*: operational taxonomic units 'OTUs_' in this study; ssp. *rubra*: 'AR_'; ssp. *tenuifolia*: 'AT_'), *A. viridis* ('AV_'), nine varieties of *Myrica rubra* (var. *biji*: 'Mbj_'; var. *baimei*: 'Mbm_'; var. *dongkui*: 'Mdk_'; var. *muye*: 'Mmy_'; var. *shuimei*: 'Msm_'; var. *wandao*: 'Mwd_'; var. *wumei*: 'Mwy_'; var. *yangliu*: 'Myl_'; var. *zaoda*: 'Mdz_'), and the ACN14a strain as an *Alnus* infection clade. Other sequences obtained from *Hippöphae salicifolia* ('Hsli_'), three *Coriaria* species (*C. myrtifolia*: 'Cm_'; *C. japonica*: 'Cj_'; *C. arborea*: 'Ca_'), *Elaeagnus angustifolia* (EAN1pec), *Datisca glomerate* and *Casuarina equisetifolia* ('CcI3') were also analyzed in the phylogeny as outgroups. These sequences were obtained from GenBank. The characters in parentheses indicated accession numbers on GenBank. Branch labels

indicate significant bootstrap values. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Fig. 3-7. Phylogenetic tree based on all the *nif*D-K sequences of *Frankia*. A maximum-likelihood phylogeny was generated based on 280 sequences of the *nif*D-K locus, obtained from three subspecies of *Alnus incana* (ssp. *hirsuta*: operational taxonomic units 'KS_' in this study; ssp. *rubra*: 'AR_'; ssp. *tenuifolia*: 'AT_'), *A. viridis* ('AV_'), nine varieties of *Myrica rubra* (var. *biji*: 'Mbj_'; var. *baimei*: 'Mbm_'; var. *dongkui*: 'Mdk_'; var. *muye*: 'Mmy_'; var. *shuimei*: 'Msm_'; var. *wandao*: 'Mwd_'; var. *wumei*: 'Mwy_'; var. *yangliu*: 'Myl_'; var. *zaoda*: 'Mdz_'), and the ACN14a strain as an *Alnus* infection clade. Other sequences obtained from *Hippöphae salicifolia* ('Hsli_'), three *Coriaria* species (*C. myrtifolia*: 'Cm_'; *C. japonica*: 'Cj_'; *C. arborea*: 'Ca_'), *Elaeagnus* ('EAN1pec'), *Datisca glomerate*, and *Casuarina equisetifolia* ('CcI3') were also included in the phylogeny as outgroups. These sequences were obtained from GenBank. The characters in parentheses indicate accession numbers on GenBank. Branch labels indicate significant bootstrap values. The tree is drawn to scale, with branch lengths measured according to the number of substitutions per site. We described OTU groups of each sequence.



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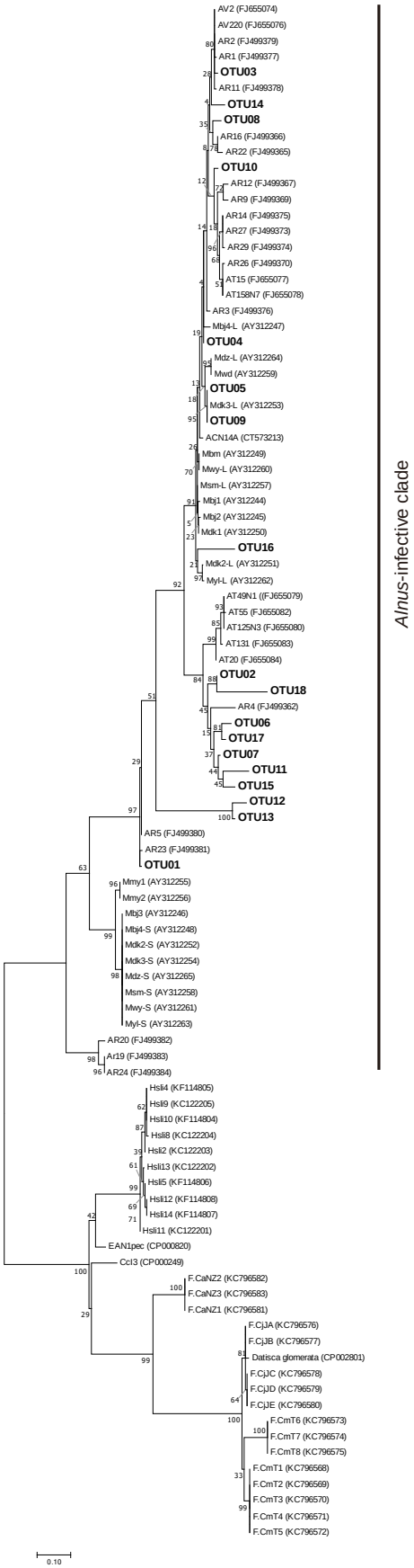
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OTU12
OTU13
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F.CmT3 (KC796570)
F.CmT4 (KC796571)
F.CmT5 (KC796572)
0.10
Alnus-infective clade

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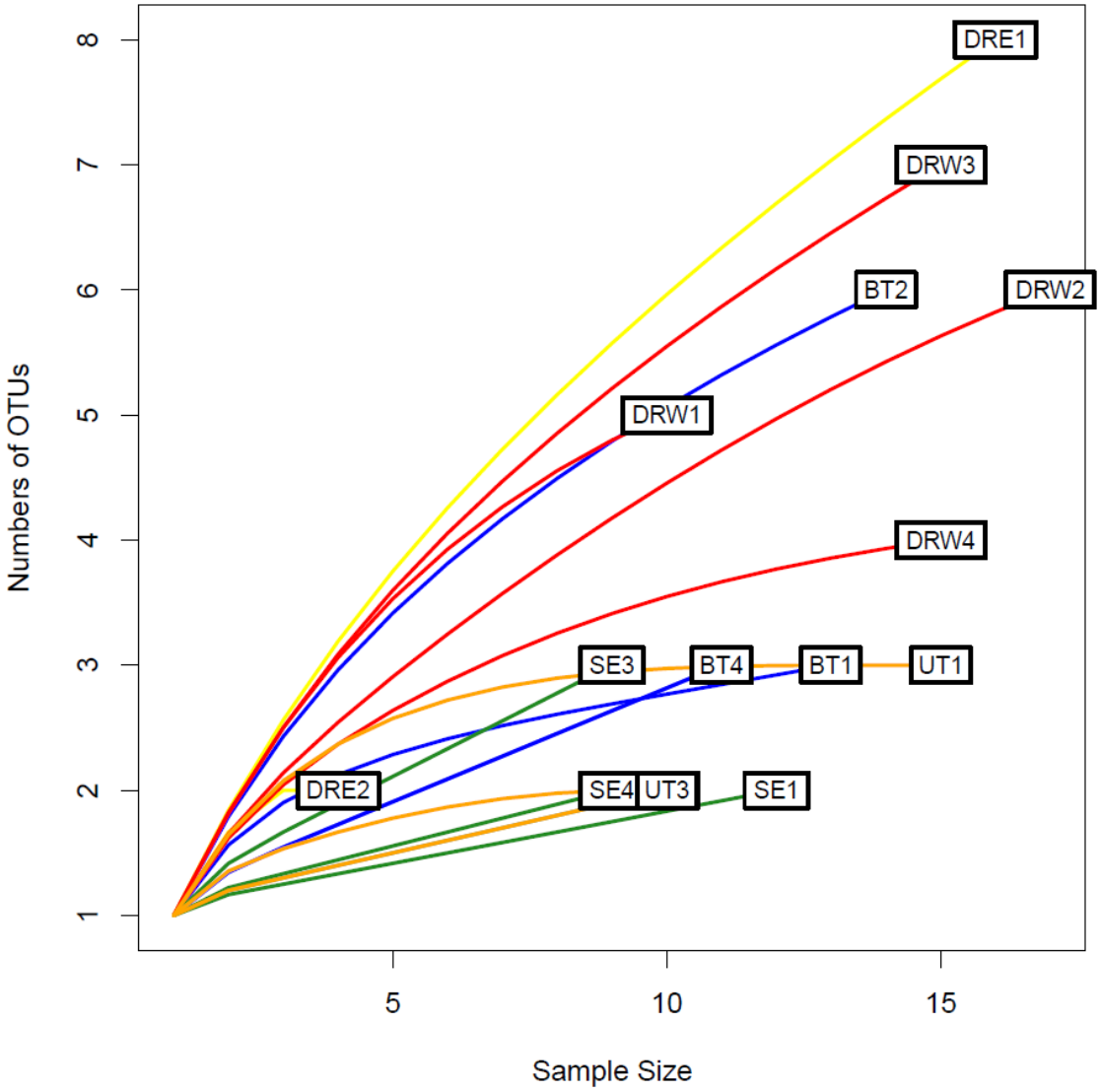


Fig. 3-3. *Frankia* diversity along sampling size in each site. Different colors indicate different areas

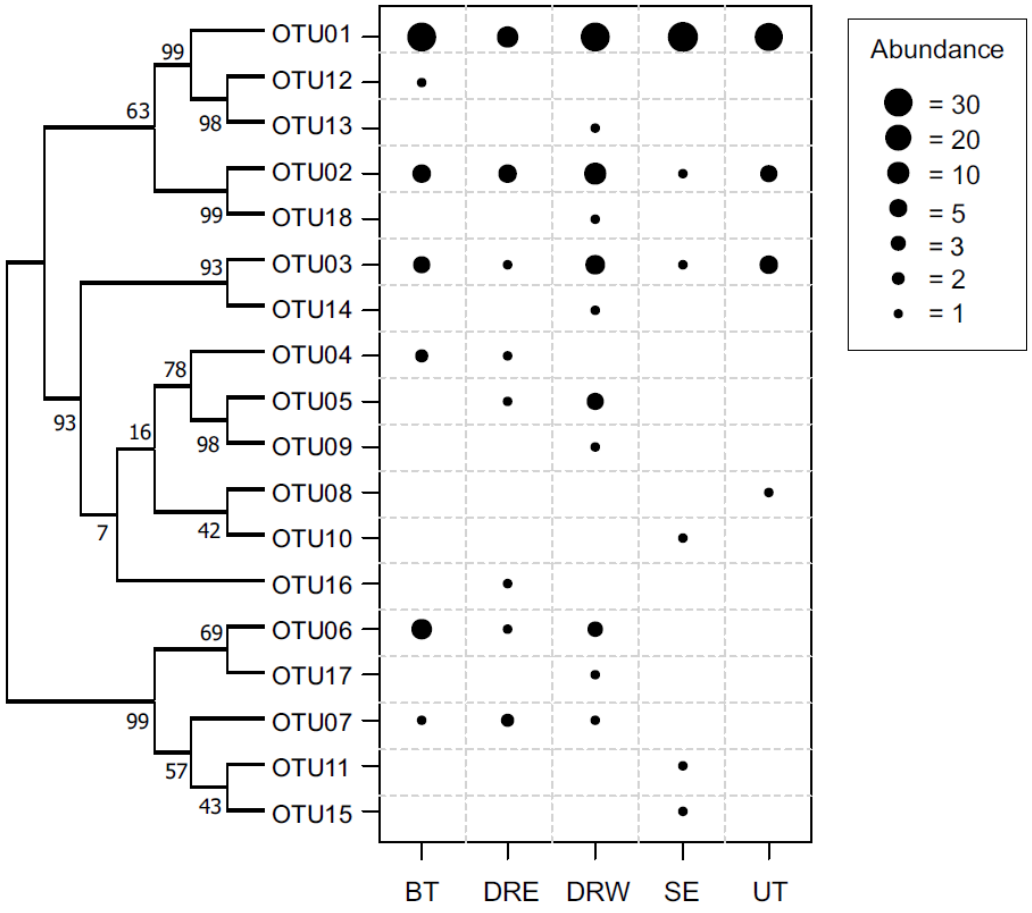


Fig. 3-4. Relationships between *Frankia* operational taxonomic units (OTUs) and areas. The phylogeny was generated using the maximum-likelihood method. Branch values indicate significant bootstrap values. Circles indicate the presence of each OTU in each site. Circle sizes represent numbers of OTUs in each site (see also Table 3-1).

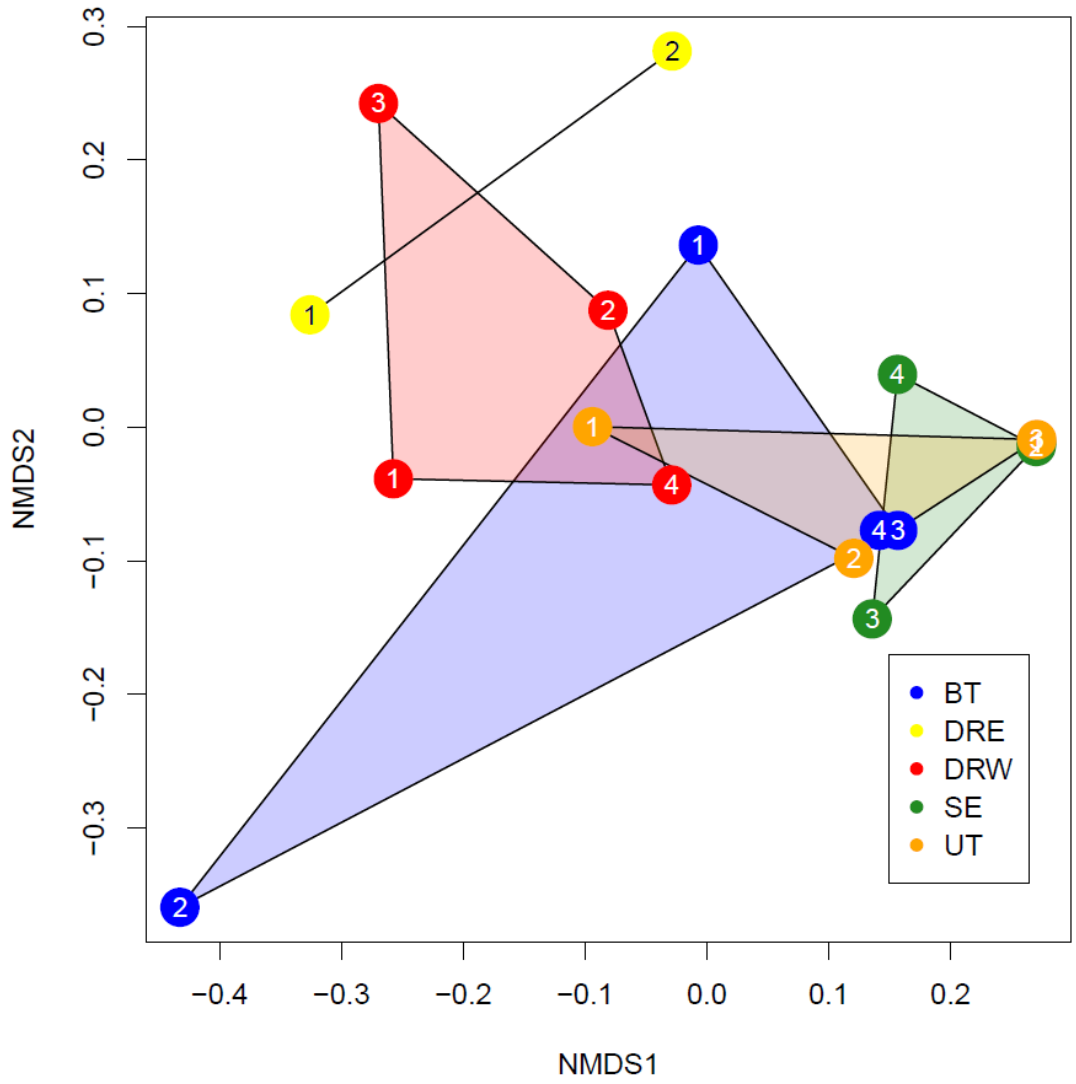
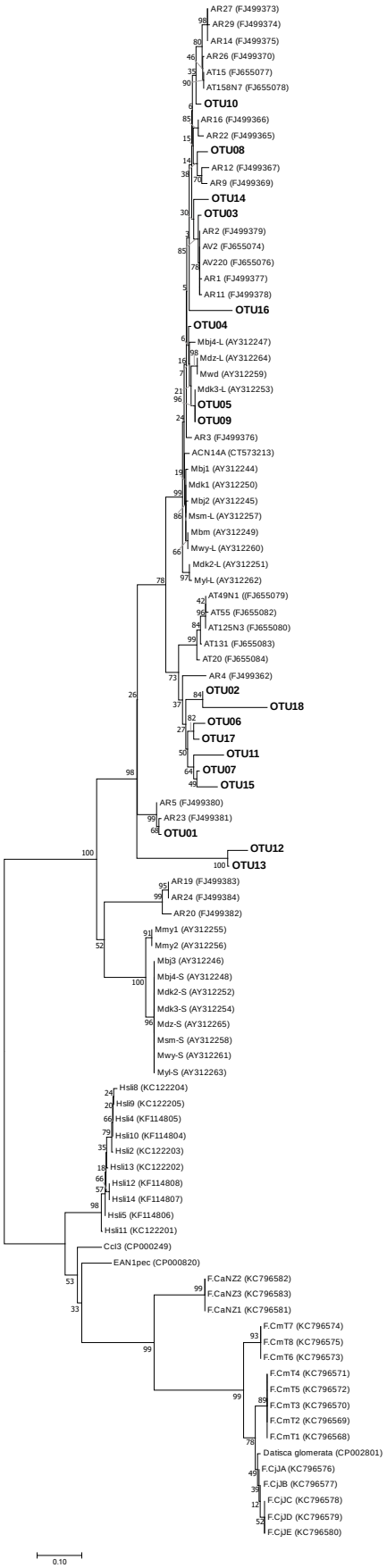


Fig. 3-5. Non-metric multidimensional scaling (NMDS) of *Frankia* OTU compositions at the site-levels. Colors of points indicate areas. The numbers in points indicate ID of sites.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AR22 (FJ499365)
OTU08
AR12 (FJ499367)
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AR2 (FJ499379)
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AR1 (FJ499377)
AR11 (FJ499378)
OTU16
OTU04
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OTU02
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F.CjJE (KC796580)
0.10

Fig. 3-6. Phylogenetic tree based on the *nif*D-K spacer region in *Frankia*. The neighbor-joining phylogeny was generated based on 97 sequences of the *nif*D-K locus, obtained from three subspecies of *Alnus incana* (ssp. *hirsuta*: operational taxonomic units 'OTUs_' in this study; ssp. *rubra*: 'AR_'; ssp. *tenuifolia*: 'AT_'), *A. viridis* ('AV_'), nine varieties of *Myrica rubra* (var. *biji*: 'Mbj_'; var. *baimei*: 'Mbm_'; var. *dongkui*: 'Mdk_'; var. *muye*: 'Mmy_'; var. *shuimei*: 'Msm_'; var. *wandao*: 'Mwd_'; var. *wumei*: 'Mwy_'; var. *yangliu*: 'Myl_'; var. *zaoda*: 'Mdz_'), and the ACN14a strain as an *Alnus* infection clade. Other sequences obtained from *Hippöphae salicifolia* ('Hsli_'), three *Coriaria* species (*C. myrtifolia*: 'Cm_'; *C. japonica*: 'Cj_'; *C. arborea*: 'Ca_'), *Elaeagnus angustifolia* (EAN1pec), *Datisca glomerate* and *Casuarina equisetifolia* ('CcI3') were also analyzed in the phylogeny as outgroups. These sequences were obtained from GenBank. The characters in parentheses indicated accession numbers on GenBank. Branch labels indicate significant bootstrap values. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

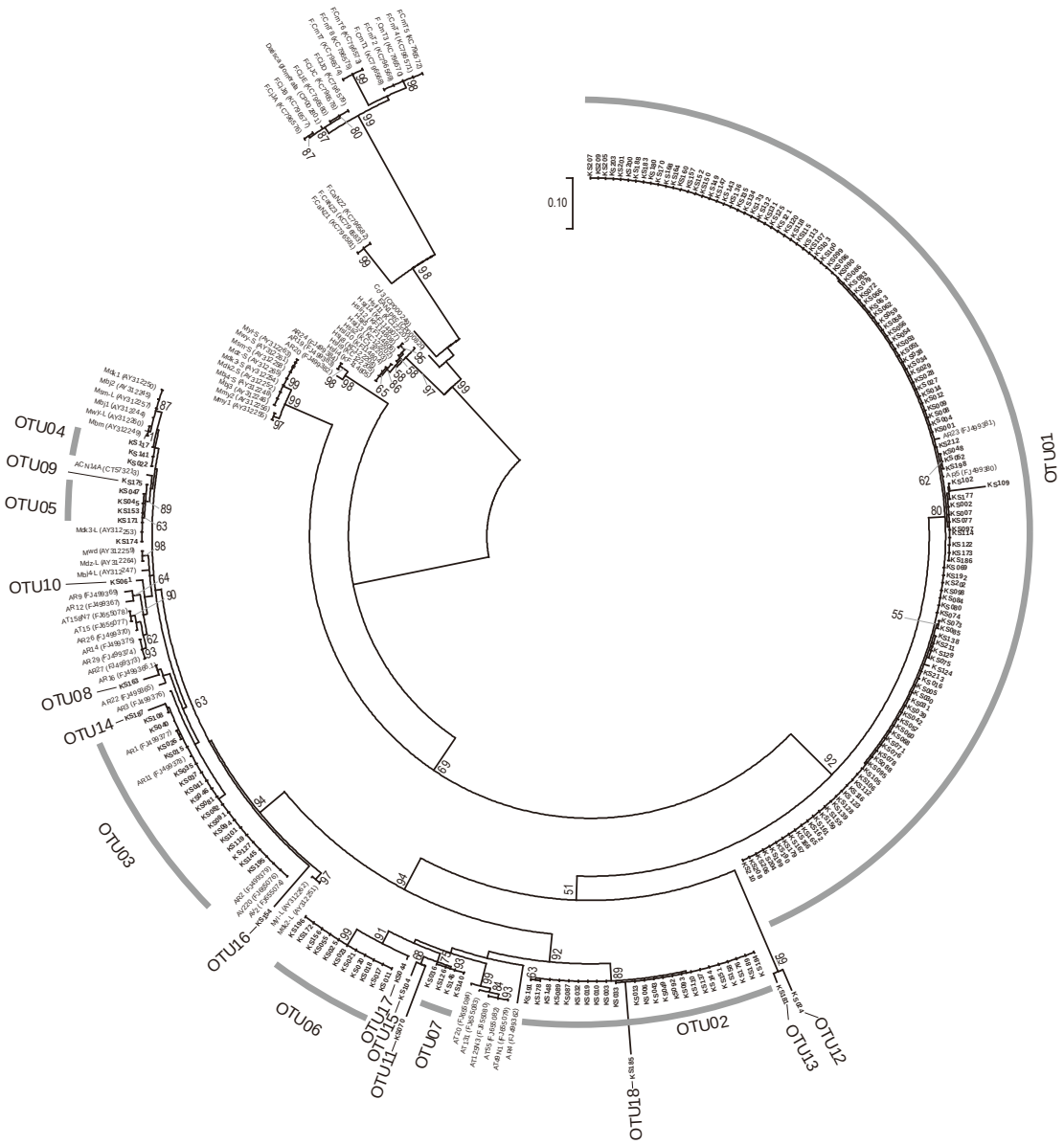

Fig. 3-7. Phylogenetic tree based on all the *nif*D-K sequences of *Frankia*. A maximum-likelihood phylogeny was generated based on 280 sequences of the *nif*D-K locus, obtained from three subspecies of *Alnus incana* (ssp. *hirsuta*: operational taxonomic units 'KS_' in this study; ssp. *rubra*: 'AR_'; ssp. *tenuifolia*: 'AT_'), *A. viridis* ('AV_'), nine varieties of *Myrica rubra* (var. *biji*: 'Mbj_'; var. *baimei*: 'Mbm_'; var. *dongkui*: 'Mdk_'; var. *muye*: 'Mmy_'; var. *shuimei*: 'Msm_'; var. *wandao*: 'Mwd_'; var. *wumei*: 'Mwy_'; var. *yangliu*: 'Myl_'; var. *zaoda*: 'Mdz_'), and the ACN14a strain as an *Alnus* infection clade. Other sequences obtained from *Hippöphae salicifolia* ('Hsli_'), three *Coriaria* species (*C. myrtifolia*: 'Cm_'; *C. japonica*: 'Cj_'; *C. arborea*: 'Ca_'), *Elaeagnus* ('EAN1pec'), *Datisca glomerate*, and *Casuarina equisetifolia* ('CcI3') were also included in the phylogeny as outgroups. These sequences were obtained from GenBank. The characters in parentheses indicate accession numbers on GenBank. Branch labels indicate significant bootstrap values. The tree is drawn to scale, with branch lengths measured according to the number of substitutions per site. We described OTU groups of each sequence.

Chapter 4. Filtering bacterial symbionts in a host plant from natural environments

Introduction

Intraspecific genetic variation has great impacts for determining biotic- and abiotic-interactions. Genetic variation in a host plant species affects a population growth rate of an herbivorous insect (Evans et al., 2012; Moreira & Mooney, 2013; Utsumi et al., 2011), and community structure such as species richness and abundance of plant-associated arthropods (Crutsinger et al., 2006; 2014; Dungey et al., 2000; Floate et al., 1997; Fritz et al., 1987; Johnson et al., 2006; Whitham et al., 1994; Wimp et al., 2005). Through these studies, the research field known as community genetics have been established.

While most of community genetics studies have focused on aboveground interactions, such as the interactions between genetic variation in host plants and herbivorous insects or arthropod communities, several studies have also revealed intraspecific genetic effects of plants on belowground communities (Schweitzer et al., 2008; 2014; 2018; terHorst & Zee, 2016). Genetic variation in plants can influence community structure in soil, such as microbial biomass and species composition, through the variation of litter decomposition (Genung et al., 2013; Lamit et al., 2015; Schweitzer et al., 2008) and allelochemicals (Evans et al., 2016; Lankau, 2011). In both above- and below-ground community genetics, a trophic interaction shaping a food web and decomposition food web is commonly a fundamental component for the linkage between plant genetics and associated communities. In this view, the concept of escape and radiate coevolution is critical (Ehrlich & Raven, 1964). This concept predicts that closely related plant species would have similar defenses and closely related herbivores would feed on closely related plants through the arms race and resultant diversification (Ehrlich & Raven, 1964; Endara et al., 2017; Futuyma & Agrawal, 2009). Similarly, community genetics studies have shown that genetically closer individuals within a plant species possess more

similar chemicals and then support more similar herbivore communities on aboveground and microbe communities in belowground (i.e., genetic similarity rule: Bangert et al., 2006; Whitham et al., 2006).

However, it is very poorly understood how communities of mutualistic symbionts are shaped by host genetic variation even though mutualistic interactions between plants and symbionts are ubiquitous components in below-ground biological interactions. Maintaining mutualistic interactions are different from trophic interactions in terms of stabilizing mechanisms. Because the persistence of interspecific cooperation needs to remove the potential advantages of cheating, the maintenance of mutualism requires stabilizing mechanism that prevents invasion of low-quality partners (Denison, 2000; Kiers & Denison, 2008; Kiers et al., 2007; Materon & Zibilske, 2001). There are several mechanisms to stabilize mutualistic partnerships: (1) partner choice, in which host plants can choose the optimal partner based on preinfection signals (Kiers & Denison, 2008), and (2) sanctions, in which host plants are monitoring mutualistic benefits from mutualistic bacteria and punish less nitrogen-fixing nodules to favor cooperation nodules (Denison, 2000; Kiers & Denison, 2008). For example, Materon & Zibilske (2001) observed that *Medicago* hosts limited infection of inefficient *Rhizobium* strains. Kiers et al. (2007) also demonstrated consistent results in soybeans–*Bradyrhizobium* symbiosis. In theoretical studies, West et al. (2002b) has shown that plants–rhizobia symbiosis becomes stable when sanctions discriminate against ineffective nodules and the sanction mechanisms were observed in empirical studies (Kiers et al., 2003). Such a stabilizing effect may constrain genetic diversity of mutualistic partners by actively favoring higher-quality partners (Archetti et al., 2011; Heath & Stinchcombe, 2014). Therefore, in contrast to escape and radiate coevolutionary process in plant–herbivore interactions, mutualistic

interactions may decrease diversity of mutualistic partners. Consequently, symbiont will be strongly filtered in community assembly on hosts and quite consistently similar across host individuals even when genetic variation in host plants is high. Incorporating symbiotic mutualisms into community genetics shed new lights on the interplay between evolutionary process and community assembly.

Frankia–Alnus mutualism provides a good opportunity to study both communities of co-infecting symbionts and non-infecting relatives in nature. Nitrogen-fixing *Frankia* bacteria form nodules on actinorhizal host plants, such as *Alnus*, *Myrica* and *Casuarina*. Because *Frankia* bacteria are also free-living microbes in soil (Benson & Dawson, 2007), this root nodule symbiosis is likely to be effective to distinguish communities of symbionts successfully infecting on hosts and communities of close relatives, which are present in soil around hosts but non-infecting. In this study, using this system, I aimed to reveal how mutualistic symbiont communities are assembled into a host plant individual from source *Frankia* communities. To address this question, I comprehensively investigated communities of mutualistic bacteria in host's root-nodules and soils in the field, using next-generation sequencing, and compare genotype composition of *Frankia*. And then, I also investigated how actinorhizal symbionts assembled into hosts are associated with genetic variation in hosts.

Methods & Materials

Host species and nitrogen-fixing bacteria

Alnus hirsuta Spach (Betulaceae) is a deciduous broadleaf tree and an early successional species. It is widely distributed in temperate riparian forests in Japan, northeastern China, Korea, and Russia. *Alnus* trees have characteristics as foundation species (Ellison et al.,

2005; 2010; Kagiya et al., 2018), which is likely to have great impacts on forest ecosystems.

Frankia sp. (Frankiaceae) are actinobacteria with the ability to convert atmospheric nitrogen gas to ammonia. *Frankia* bacteria are free-living microbes in soil (Benson & Dawson, 2007). While *Frankia* that form nodules on roots of phylogenetically diverse host plants are gram-positive, rhizobia that form nodules on roots of legume plants are gram-negative.

Study sites

Our study sites are located in and around Uryu experimental forest (44°030-290N, 142°010-200E; Chapter 2, 3). This experimental forest is natural conifer-broadleaf mixed forest. Subsequent sampling was conducted in 12 sites along 4 river areas where the investigation in Chapter 2 was performed. There are only *A. hirsuta* as actinorhizal plants in these sites.

Root nodules, rhizosphere soils, and riparian soils sampling

In the study sites, I collected root nodules, rhizosphere soils (i.e., inside under host tree crown), and riparian soils (i.e., riparian but outside of alder crown). Root nodules were collected from roots of 78 adult trees of the alders, which were used in my previous study (Kagiya et al., 2018; see Chapter 2) because I have genomic information for these trees. The nodules were collected from seven roots per alder individuals based on the result in Chapter 3 that seven *Frankia* OTUs were detected from multiple seedlings of 201 alder seedlings in the whole of this forest. The sampling was performed in August 2017.

Rhizosphere soils were collected from August to September 2018. Following litter layer removal, I collected a 10 × 10 × 10 cm soil block from inside area under each of the 78 alders' crowns, using a shovel. I randomly collected three soil blocks under each tree crown. The shovels were sterilized at 121°C for 20 minutes before sampling, and disinfected by 70% (v/v) ethanol after every soil sampling. I were changed shovels to new ones when I moved to different sites.

To sample riparian soils, I established 40 m transects along the rivers at the centroid points of the studied alder individuals within each of study sites. The riparian soils samplings were conducted at every 10 m along the transects (i.e., four points in total per each site). At the points, I collected the 10 cm^3 soil blocks in the same way of rhizosphere soil samplings. The riparian soils samplings were performed from July to August in 2017. From these samplings, I obtained total of 12 riparian soil samples. To investigate environmental conditions, at the sampling points, soil pH was measured using direct soil pH probe kit (Hanna). After bringing soils to laboratory, total inorganic nitrogen, including NH_4^+ and NO_3^-, was extracted from 10 g of the sampled riparian soils by 100 mL of 2M KCl, and analyzed with the auto analyzer (AACS-4, BL-TEC Inc., Japan; the detailed method of total inorganic nitrogen analysis is described in Fukuzawa et al., 2006).

Frankia DNA extraction from root nodules and soils

The collected nodule and soil samples were transported to a laboratory in a cool box, and then immediately extracted *Frankia* DNA. The root nodules sterilized using 10% (v/v) Clorox bleach. The one lobe was picked from each washed nodule, sliced using sterilized razor blades, and crushed using sterilized homogenization sticks. The crushed lobes were at 37 °C for 60 minutes with 25 μL Proteinase K. DNA was extracted from the crushed

lobes using DNeasy Blood & Tissue Kit (Qiagen). DNA was extracted from 300 mg of the sampled rhizosphere and riparian soils using NucleoSpin Soil (Macherey-Nagel).

Library preparation

For rhizosphere and riparian soil samples, a nested polymerase chain reaction (PCR) was performed to amplify a *nif*D-K fragment in the samples because in the amount of *Frankia* DNA in soil is very low so that sufficient amounts of *Frankia* DNA needed for NGS sequencing are not obtained by single PCR (Ben Tekaya et al., 2018; Rodriguez et al., 2016). A 496-bp fragment of *nif*D-K IGS region was amplified by the PCR approach with the *Frankia*-specific primer pair, *nif*D1310frGC (5'-CGC CAG ATG CAC TCC TGG GAC TAC T-3') and nifKR331frGC (5'-CGG GCG AAG TGG CTG CGG AA-3'). PCR amplification was performed as follows: one cycle at 95 °C for 2 min, followed by 35 cycles of 95 °C for one min and 64 °C for 5min, and a final step of one cycle at 72 °C for 5 min.

Using these successful PCR productions and extracted DNA from host nodules, the subsequent PCR was performed to amplify a 353-bp fragment of *nif*D-K intergenic spacer region with the *Frankia*-specific primer pair, *nif*D13fw-NexteraTailed (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNN NNN GGA CTA CTC CGG GCC CTA C-3') and nifK418rv-NexteraTailed (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GNN NNN NAG ATG TCG TTG TGG TCG AG-3'). This process is as the first stage PCR for the usual NGS library preparation workflow. PCR amplification was performed as follows: one cycle at 95 °C for 2 min, followed by 35 cycles of 95 °C for one min and 61 °C for 5min, and a final step of one cycle at 72 °C for 5 min. All successful PCR products were cleaned using AMPure XP magnetic beads

(BECKMAN COULTER). These cleaned products were tagged by unique indices per sample performing index PCR with Nextera XT Index 1 and 2 primers (Illumina) as the second stage PCR. Index PCR was performed as follows: one cycle at 95 °C for 3 min, followed by 8 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec, and a final step of one cycle at 72 °C for 5 min. After the index PCR, the products were cleaned by the AMPure magnetic beads again, and then libraries with the same amplicon concentration are pooled. Pooled library was quantified by qPCR, using library quantification kit (Takara Bio).

These samples were analyzed, using Illumina MiSeq with paired-end 300 bp reads (Macrogen, Korea).

Bioinformatics

Obtained paired-end reads filtered and processed using DADA2 pipeline (Callahan et al., 2016) in QIIME2-2019.01 (Bolyen et al., 2019). For soil samples, sequences representing < 1% of the total numbers of reads per sample were removed using the resulting BIOM table and a customized R script (Ben Tekaya et al., 2018; Rodriguez et al., 2016) because these samples were amplified on the nested PCR. For nodule samples, the samples that included numbers of reads within 99 percentiles of total number of all sample reads were allowed to use in subsequent analysis (n = 72). Then, I obtained amplicon sequence variants (ASVs). The sequences were interrogated with BLAST+ 2.9.0 blastn (Camacho et al., 2009) to remove non-*Frankia* sequences. These sequences were also clustered on Operational taxonomic units (OTUs) separation with sequences of 18 uncultured *Frankia* strains (i.e., OTU01-18 strains defined as the accession number of LC482655-LC482672; Chapter 3) as references. OTUs separation was performed using the CD-Hit program (Li

& Godzik, 2006) at 97.0 % similarity threshold. This threshold was decided based on Põlme et al. (2014) and Chapter 3.

Phylogenetic trees were constructed using Maximum Likelihood (ML; bootstrap analyses with 1000 replications) based on the Kimura 2-parameter evolutionary model (Kimura, 1980) with a discrete gamma distribution, which was selected by evolutionary model selection procedure in MEGA 7.0.21 (Kumar et al., 2016). The phylogeny included the *nif*D-K locus of uncultured *Frankia* bacteria obtained from two *Alnus* species, *A. incana* (ssp. *tenuifolia*; Anderson et al., 2009 and ssp. rubra) and *A. viridis* (Anderson et al. 2009), and nine varieties of *Myrica rubra* (var. *biji,* var. *baimei,* var. *dongkui,* var. *muye,* var. *shuimei,* var. *wandao,* var. *wumei,* var. *yangliu,* var. *zaoda*; He, Chen, Hu, & Asghar, 2004), as well as a cultured ACN14a strain, whose host is *A. viridis* (Normand et al., 2007). Other *Frankia* sequences from different actinorhizal species, *Hippöphae salicifolia* (Mishra et al. 2015), three *Coriaria* species (*C. myrtifolia, C. japonica, C. arborea*; Nouioui et al. 2014), *Elaeagnus angustifolia*, *Datisca glomerate*, and *Casuarina equisetifolia* (Normand et al. 2007), were also included as outgroups. These sequences covered *Frankia nif*D-K sequences of almost actinorhizal hosts in GenBank. These sequences were obtained from GenBank. All positions with less than 95% site coverage were eliminated. Both ML and NJ phylogenetic trees were generated with MEGA 7.0.21 (Kumar et al., 2016).

Community analysis

The community data of *Frankia* OTUs was standardized by coverage-based rarefaction (Chao & Jost, 2012) and transformed to presence/absence data. Non-metric multidimensional scaling (NMDS) was performed to visualize *Frankia* community

dissimilarity in this study.

To elucidate whether filtering force drives symbiont assembly into host plants, I generated 1000 null models of symbiont community by seven random samplings from overall rhizosphere source community with frequency distribution of OTUs in rhizosphere soil. I compared *Frankia* OTU richness and composition in host's root-nodules with the null model, using generalized linear model (GLM) with Poisson distribution and permutation MANOVA (PERMANOVA) with 9999 permutations, using the lme4 1.1-21 package (Bates et al., 2019) and the vegan 2.5-5 package of the R 3.6.1 software, respectively.

To quantify variation in symbiont filtering force among hosts, I defined "symbiosis filtering" index as dissimilarity in *Frankia* community composition between roots-nodules and those in rhizosphere soils in each river area. This symbiosis filtering index indicates how some *Frankia* genotypes were filtered some *Frankia* genotypes from a genetic pool in each river area. In order to quantify differences in symbiosis filtering index, I firstly calculated symbiosis filtering indices as Jaccard dissimilarities between root-nodules of host individuals and rhizosphere soils in each river area, using 56 individuals of studied alders which had both *Frankia* community data in root nodules and in rhizosphere soils. Secondly, the above symbiosis filtering indices were averaged for each site. Thirdly, Euclidian distance of the site-level symbiosis filtering indices was calculated among sites, I referred to the distance as "symbiosis filtering differences". In addition, to estimate the genetic distance among sites, I calculated Nei's genetic distance (Nei, 1978), based on obtained 1077 SNPs by RAD-Seq (Kagiya et al., 2018; in Chapter 2). Finally, the correlation between the Nei's genetic distance of alders and the symbiosis filtering differences was examined, using Mantel tests. All of the above community

analyses were performed with the vegan 2.5-5 package in the R software 3.6.1.

To quantify the relative contribution of genetic distance of hosts and other abiotic factors in explaining the spatial patterns of symbiosis filtering with accounting for spatial autocorrelation, generalized dissimilarity modelling (GDM; Blois et al., 2013; Capinha et al., 2015; Ferrier et al., 2007; Lasram et al., 2015) was performed. GDM is an I-spline-based extension of matrix regression that accounts for nonlinearities between explanatory variables and community dissimilarity. As GDM uses flexible splines, it is capable of fitting nonlinearity relationships between community dissimilarity and environmental variables (Ferrier et al., 2007). GDM models for the symbiosis filtering differences included Nei's genetic distance of hosts, soil pH, and the total amount of inorganic nitrogen in soils, Jaccard community dissimilarity of *Frankia* in rhizosphere soils, and spatial distance as predictors. To test whether the genetic distance of alders could explain the symbiosis filtering differences without other factors, I estimated unique and shared contribution of each predictor to the total deviance explained by partitioning the predictors with GDM models (Capinha et al., 2015; Fitzpatrick et al., 2013; Kagiya et al., 2018). To calculate the unique contribution of genetic distance and other factors, the deviance explained of the model removed each predictor was subtracted from the deviance explained of the full model. The results were converted into percentages where the deviance explained of the full model was 100%. To calculate the shared contributions of both predictors, a value of deviance explained of the model that was fitted by both predictors was subtracted unique contributions of each predictor.

Results

Genetic composition of symbiotic and free-living *Frankia* communities

I obtained 352 ASVs in total from 138 samples consisted of riparian soil, rhizosphere soil, and root nodules (Fig. 4-1). 90.34 % of ASVs is unique singleton variants, which occurred once in 138 samples. I found profoundly diverse ASVs from rhizosphere and riparian soils (rhizosphere: 228 ASVs, n = 54; riparian: 103 ASVs, n = 12). In contrast, in root nodules, the total number of ASVs was quite limited (38 ASVs, n = 72). These results suggest that the *Frankia* composition in root nodules are constructed by limited members across host individuals.

To classify *Frankia* genetic variants in this study, I used OTU methods based on the genetic similarity of the *nif*D-K loci. A total of 19 OTUs were obtained at a 97.0% similarity threshold. Consequently, most ASVs from root nodules and riparian soils converged to a few but same OTUs (OTU01, 02, and 03), while a variety of OTUs were still found in rhizosphere soil (Fig. 4-2). In consistent, sample-based rarefaction curves showed that the number of *Frankia* OTUs did not completely reach to saturation but was significantly greater in rhizosphere than root nodules after standardizing sample size (Fig. 4-3). In addition, the number of *Frankia* OTUs in riparian soils was lower than the number of OTUs in rhizosphere soils (Fig. 4-3). These findings suggest that the occurrence of host plants promote the local assemblage of nitrogen-fixing bacteria in soil rather than homogeneity dispersion of diverse bacterial strains in a natural forest.

Moreover, the phylogenetic trees indicated that *Frankia* OTUs detected in my study covered a wide variety of *Alnus*-infecting strains reported so far (Fig. 4-4). Most abundant strains (i.e., OTU01, 02, and 03) belonged to phylogenetically distant clades (Fig 4-4), indicating that the *Alnus* host population accepts genetically diverse bacterial symbionts.

Filtering force for *Frankia* assembly on host's root-nodules

Frankia composition of root-nodule community was significantly different from the composition of rhizosphere community (PERMANOVA: $P < 0.001$). Community variance of *Frankia* in rhizosphere soils was greater than the variance of community in host root–nodules (PERMDISP: $P < 0.001$; Fig. 4-5). The communities of *Frankia* in hosts' root–nodules were constructed by a small part of overall members in communities of rhizosphere soils (8/16 OTUs were detected from both nodules and rhizosphere soils). Furthermore, rank–abundance dominance plots represented that three OTUs (i.e., OTU01,02, and 03) were ultimately dominated and abundance decay was very steep in root nodules (Fig. 4-6). However, dominance was not obvious and abundance decay was gradual in rhizosphere communities (Fig. 4-6). These results showed that a host plant associated with limited members from diverse resource of symbiont community in soil.

The number of *Frankia* OTUs from root nodules was significantly lower than expected OTU richness by random sampling from rhizosphere composition (Fig 4-7). Moreover, significantly different *Frankia* composition was constructed in root nodules with the composition in the null model (PERMANOVA: $P < 0.001$). These results provide evidence that filtering force works to shape symbiont communities on the host plant with strains in narrow ranges than expected from *Frankia* pools. Furthermore, no correlations between the dissimilarity of *Frankia* communities on host roots and Nei's genetic distance of hosts (Mantel tests: $r = -0.3093$, $P = 0.9033$), supporting my hypothesis that community composition in microsymbionts is consistent across host individuals despite genetic variation in hosts.

Host's genetic variation in symbiosis filtering

Symbiosis filtering differences were significantly correlated with hosts' genetic distance (Fig 4-8a). This correlation remained significant even when spatial distance among alder individuals was corrected using a partial Mantel test ($r = 0.444$, $P < 0.001$). Furthermore, GDM analysis quantified that the explanatory proportions of genetic distance of the hosts and other predictors, including spatial distance among hosts, soil pH, amount of inorganic nitrogen in soils, and community dissimilarity of *Frankia* in rhizosphere soils, for symbiosis filtering differences (Fig 4-8b). The genetic distance of alders and other factors, including geographic distance of the alders, soil pH, the total amount of inorganic nitrogen in soils, and community dissimilarity of *Frankia* in soils, absolutely explained 36.64% of deviance in the symbiosis filtering differences.

Where the deviance explained by the model with genetic distance and other factors was 100 %, the genetic distance of alders uniquely explained 47.94% of the deviance while the other factors uniquely explained 1.31%. This result demonstrated that genetic distance of the hosts was the best of measured predictors for the symbiosis filtering differences.

These findings suggested that differences in symbiosis filtering force among host individuals could generate slight differences of symbiont composition among host individuals in order to alter obtained members of mutualistic bacteria from rhizosphere soils, while symbiosis filtering might work to preserve high homogeneity of symbiont composition in host individuals. Moreover, the differences in symbiosis filtering force were likely to covary with genetic variation of host plants rather than soil environments surrounding the hosts.

Discussion

This study comprehensively investigated genetic diversity and composition of *Frankia* in different environments, using next-generation sequencing. The occurrence of host plant individuals promoted the local assemblage of diverse *Frankia* strains and the greater ASV and OTU richness of *Frankia* was detected in rhizosphere soils around hosts than root nodules and host-absent riparian soil. *Frankia* composition in root-nodules consisted of a few members (Fig 4-2). Because *Frankia* richness in root-nodules was significantly lower than the null model, my findings represented that the members of symbionts within hosts were selected from surrounding pools by filtering force. Moreover, the community dissimilarity approach suggests that genetic variation in host plants underlies the symbiosis filtering difference.

Filtering of mutualistic partnerships in the field

This study found similar symbiotic composition among genetic variation in a host tree contrary to prediction in genetic similarity rule (Bangert et al., 2006). A lot of theoretical and empirical studies have shown that stabilizing mechanisms maintain mutualistic interactions under environments with high diversity of mutualistic partners, including cheaters (Archetti & Scheuring, 2013; Archetti et al., 2011; Foster & Kokko, 2006; Hoeksema & Kummel, 2003; Kiers & Denison, 2008; Kiers et al., 2007, 2003; Law & Koptur, 1986; Materon & Zibilske, 2001; McNamara & Leimar, 2010; West et al., 2002ab; Weyl et al., 2010). In my findings, *Frankia* communities in host individuals were constructed by smaller numbers of *Frankia* OTUs than the number of *Frankia* OTUs in surrounding environments (Fig. 4-1, 4-2, 4-3). Moreover, the comparison with the null models showed that the *Frankia* communities in the host plant were not chosen from soils by chance (Fig. 4-7). This filtering effect on bacterial assembly might be resulted from

stabilizing mechanisms that were predicted to reduce genetic diversity of symbionts.

While filtering force were likely to regulate symbiont assembly into a host plant in a natural system, most alders interacted with phylogenetically different OTUs (i.e., OTU01, 02, and 03; Fig 4-2, 4). This may be resulted from genetic variation in bacterial functions. Dean et al. (2014) found that nitrogen resources from nitrogen-fixing symbionts were needed for induced herbivory resistance and artificial fertilizer did not induce the resistance. This suggests that herbivore resistance does not depend on the amount of total nitrogen supply but on specific nitrogen forms, such as amides and ureides, provided by nitrogen-fixing symbionts. Nitrogen fixed by associated rhizobacteria, including *Frankia*, can be stored in nodules and transported to aerial parts as these specific forms (Berry et al., 2011; Dean et al, 2009; Schubert, 1986).

Nitrogen fixation is the fundamental function of nodulating bacteria including *Frankia* bacteria, promoting the performance of host plants such as growth, survival and herbivory defense. However, *Frankia* bacteria have some other plant growth promoting functions, such as solubilization of inorganic phosphate (Sayed et al., 2002) and syntheses of plant hormones (Péret et al., 2007) and siderophores (Tisa et al., 2016). Nouioui et al. (2019) discovered genes encoding these functions from some *Frankia* strains *in silico* genome analyses, and also detected variation in the genes among the *Frankia* strains.

The symbioses with genetically diverse microsymbionts could be explained by complimentary effects of genetic variation in these multi-functions of *Frankia* strains on the hosts' performances.

Assembly of bacterial symbionts for rhizosphere soil under host plants

Numerous *Frankia* ASVs were detected from both rhizosphere and riparian soils while

numbers of ASVs from root nodules were limited (Fig 4-1). *Frankia* communities were more diverse in rhizosphere than the communities in nodules and in riparian soils even when *Frankia* sequences were separated by OTUs (Fig 4-2). This finding indicated that phylogenetically distant genotypes assembled in host rhizosphere soil.

The reason why higher *Frankia* diversity was found in rhizosphere soils than in riparian soils can be explained by the occurrence of hosts. In rhizosphere soil, free-living *Frankia* obtains carbon sources from plant root exudates (Chaia et al., 2010; Rönkkö et al., 1993; Samant et al., 2015; 2016; Smolander et al., 1990; Valdés, 2008). In addition, secondary metabolites in root exudates such as flavonoids act as signals in development of plant-microbe symbiosis associations, including actinorhizal symbioses (Hughes et al., 1999; Oldroyd & Downie, 2008; Perrine-Walker et al., 2011; Steinkellner et al., 2007; Taylor & Grotewold, 2005; Vessey et al., Pawlowski & Bergman, 2005; Woo et al., 2005).

These chemical compounds in host's root exudates may promote the local assembly of *Frankia* in host rhizosphere, regardless of infecting and uninfecting types.

Genetic variation in filtering force of mutualistic partnerships

Despite symbiosis filtering to favor the specific genotypes, genetic diversity of *Frankia* was high in rhizosphere soil under a host plant. Consistently, a lot of previous studies found genetic variation in mutualistic partners are sustained in nature (Barrett et al, 2015; 2016; Bever et al., 2013; Clawson et al., 1998; 1999; Huguet et al., 2001; Põlme et al., 2014; Ridgway et al., 2004; Shiraishi et al., 2011; Young et al., 1987). However, stabilizing mechanisms alone cannot explain the persistence of the variation because the evolutionary fixation of the most beneficial partner genotypes is predicted through the stabilization (Heath & Stinchcombe, 2014; Law & Koptur, 1986; Parker, 1999; Weyl et

al., 2010). Furthermore, the fixation of the beneficial genotypes may lead to the loss of discrimination ability by hosts if it needs a cost (Foster & Kokko, 2006; Frederickson, 2013; Heath & Tiffin, 2009; McNamara & Leimar, 2010). One solution to this paradox is mutation–selection balance (Heath & Stinchcombe, 2014). It is the hypothesis that variation in partner quality is maintained by the balances between mutation which introduces genotypes of lower quality and selection which acts to favor the partner genotypes of higher quality. Thus, estimating the strength of selection that stabilizing mechanisms impose on partner quality in nature would help us toward an understanding of evolution of partner quality (Heath & Stinchcombe, 2014).

This study quantified the symbiosis filtering force using community dissimilarity approach and found the possibility of genetic variation of hosts in the filtering force in the field (Fig 4-8). The reason why similar bacterial compositions were sustained among almost alders in spite of the variation in the filtering force among host individuals is likely to be explained by spatial heterogeneity in genetic diversity of *Frankia*. *Frankia* genotypes heterogeneously dispersed in the forest (Chapter 3). NMDS also showed that high variation of *Frankia* composition in rhizosphere soils among host individuals (Fig. 4-5). The necessity of filtering force could depend on genetic diversity and/or relative abundance of *Frankia* genotypes in surrounding local soil. If the ability of filtering requires costs and a common optimal symbiont composition is consistent among host individuals, the balance between the necessity and costs can maintain the homogeneity of symbiont composition in a host plant along with genetic variation in filtering force.

The variation in filtering force might also contribute to the maintenance of genetic variation in microsymbionts. When variation in stabilizing mechanisms alters selection pressure for microsymbionts, genetic variation in microsymbionts, even

including lower quality genotypes, may be sustained (Heath & Stinchcombe, 2014). For example, some studies reported that sanctions were not perfectly effective because they cannot respond to nonzero benefit levels or cannot punish individual partner genotypes (Charlotte et al., 2012; Kiers et al., 2006). If genetic variation in a host plant alters strength of symbiosis filtering, genetic variation in the host will be the key to reveal coevolution in mutualistic interactions under natural ecosystems with high genetic diversity of microsymbionts.

The present study clearly demonstrated that host plants interacted specific symbiont genotypes through symbiosis filtering, which might be resulted from stabilizing mechanisms in mutualism such as partner choices and sanctions, in the field. My finding that symbiosis filtering indices correlated with genetic distance of hosts is likely to be evidence of linkage between host genetics and symbiont assembly in nature even if symbiosis filtering indices relatively depend on dissimilarity of *Frankia* composition in rhizosphere soils among rivers and/or uninfective strains construct *Frankia* communities in rhizosphere soils. Future studies should pay attentions to difference in partner quality and infectivity between infecting and non-infecting members of communities to understand mutualistic coevolution in natural ecosystems. Moreover, revealing genetic basis underlying stabilizing mechanisms is fruitful toward an understanding of the interplay between coevolutionary dynamics in mutualism and community assembly in symbiosis.

Figure legends

Fig. 4-1. Amplicon sequence variants (ASVs) composition of *Frankia* in host' root-nodules (a) and soils (b). (b) gray area represents ASVs from non-host soils and white area represents ASVs from host rhizosphere soils. Colors indicates each river area (blue: BT; red: DR; green: SE; yellow: UT).

Fig. 4-2. *Frankia* OTUs compositions in *Alnus* host individuals (a) and soils (b). (b) gray area represents OTUs from non-host soils and white area represents OTUs from host rhizosphere soils. Colors indicates each river area (blue: BT; red: DR; green: SE; yellow: UT).

Fig. 4-3. Sample-based rarefaction curves of *Frankia* OTUs with ranges of SD of each sample type. Line colors represent sample types: nodule community (green), rhizosphere soil community (yellow), and riparian soil community (red).

Fig. 4-4. Phylogenetic tree based on the *nif*D-K spacer region in *Frankia*. A maximum-likelihood phylogeny was generated based on 110 sequences of the *nif*D-K locus, obtained from three subspecies of *Alnus incana* (ssp. *hirsuta*: operational taxonomic units 'OTUs_' in this study; ssp. *rubra*: 'AR_'; ssp. *tenuifolia*: 'AT_'), *A. viridis* ('AV_'), nine varieties of *Myrica rubra* (var. *biji*: 'Mbj_'; var. *baimei*: 'Mbm_'; var. *dongkui*: 'Mdk_'; var. *muye*: 'Mmy_'; var. *shuimei*: 'Msm_'; var. *wandao*: 'Mwd_'; var. *wumei*: 'Mwy_'; var. *yangliu*: 'Myl_'; var. *zaoda*: 'Mdz_'), and the ACN14a strain as an *Alnus* infection clade. Other sequences obtained from *Hippöphae salicifolia* ('Hsli_'), three *Coriaria* species (*C. myrtifolia*: 'Cm_'; *C. japonica*: 'Cj_'; *C. arborea*: 'Ca_'), *Elaeagnus* ('EAN1pec'), *Datisca glomerate*, and *Casuarina equisetifolia* ('CcI3') were also included in the phylogeny as outgroups. These sequences were obtained from GenBank. The characters in parentheses indicate accession numbers on GenBank. Branch labels indicate significant bootstrap values. The tree is drawn to scale, with branch lengths measured according to the number of substitutions per site. Colors of OTU labels indicate sample types (blue: from both nodules and soils; red: from only soil; green: from only nodules; black: from only seedlings in Chapter 3).

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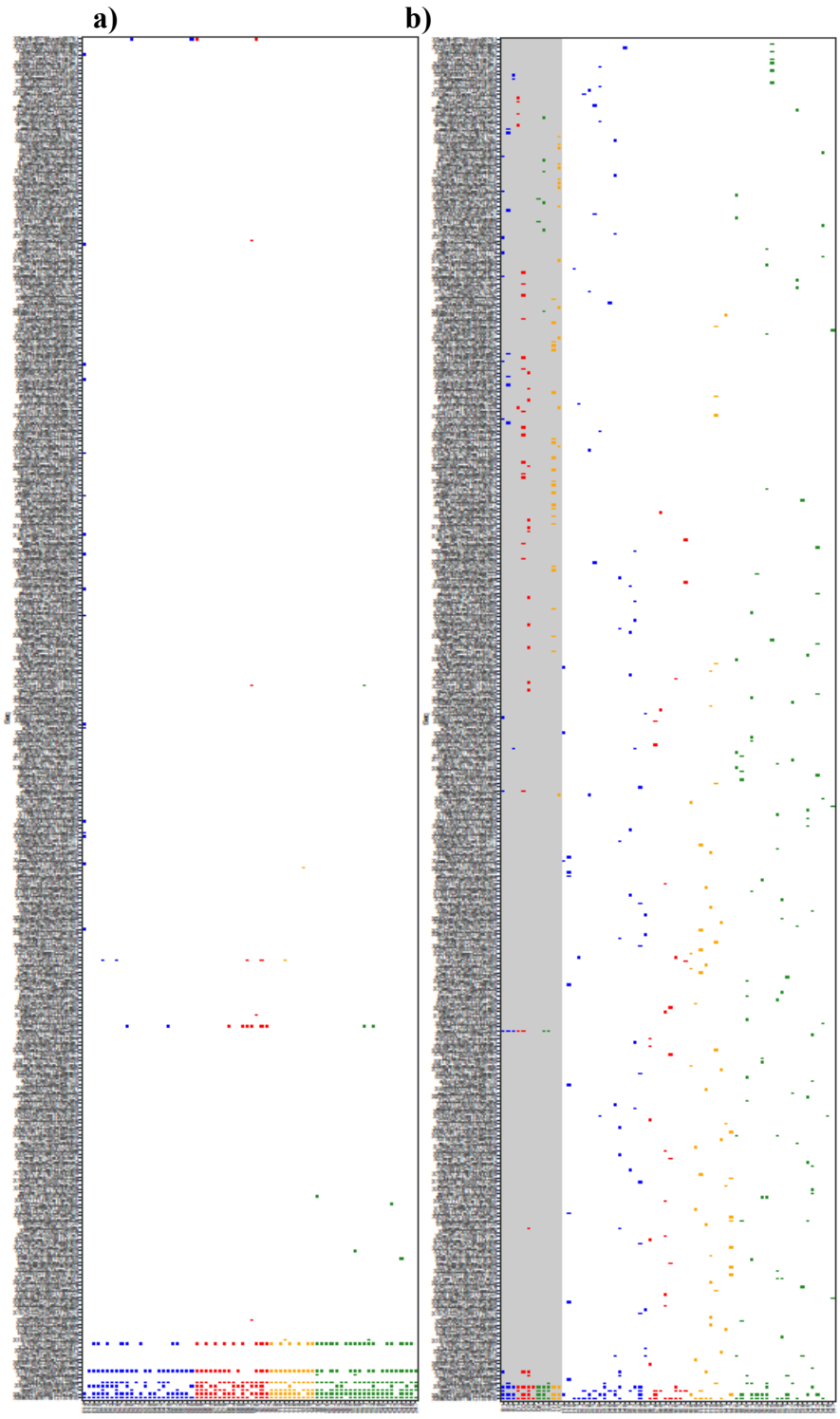
Figures



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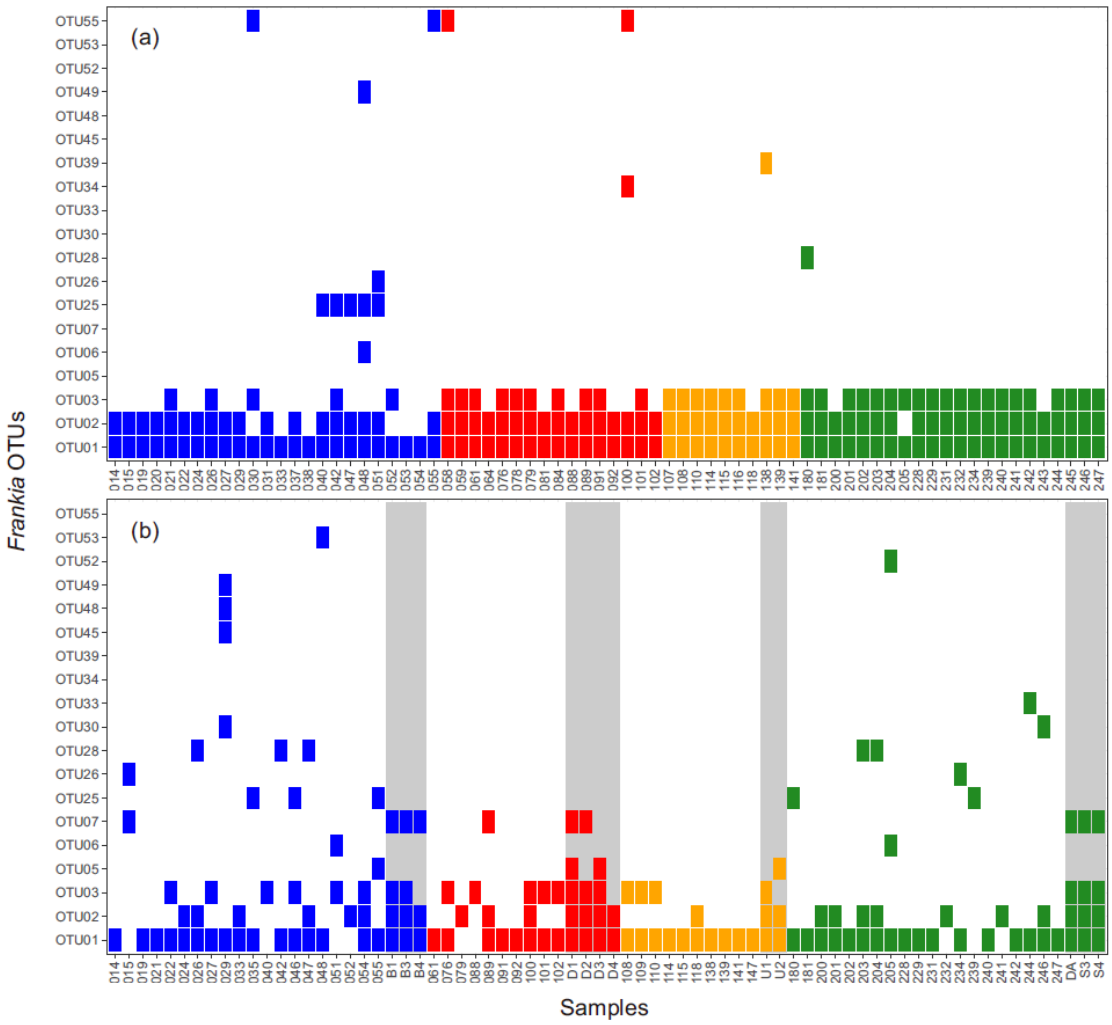

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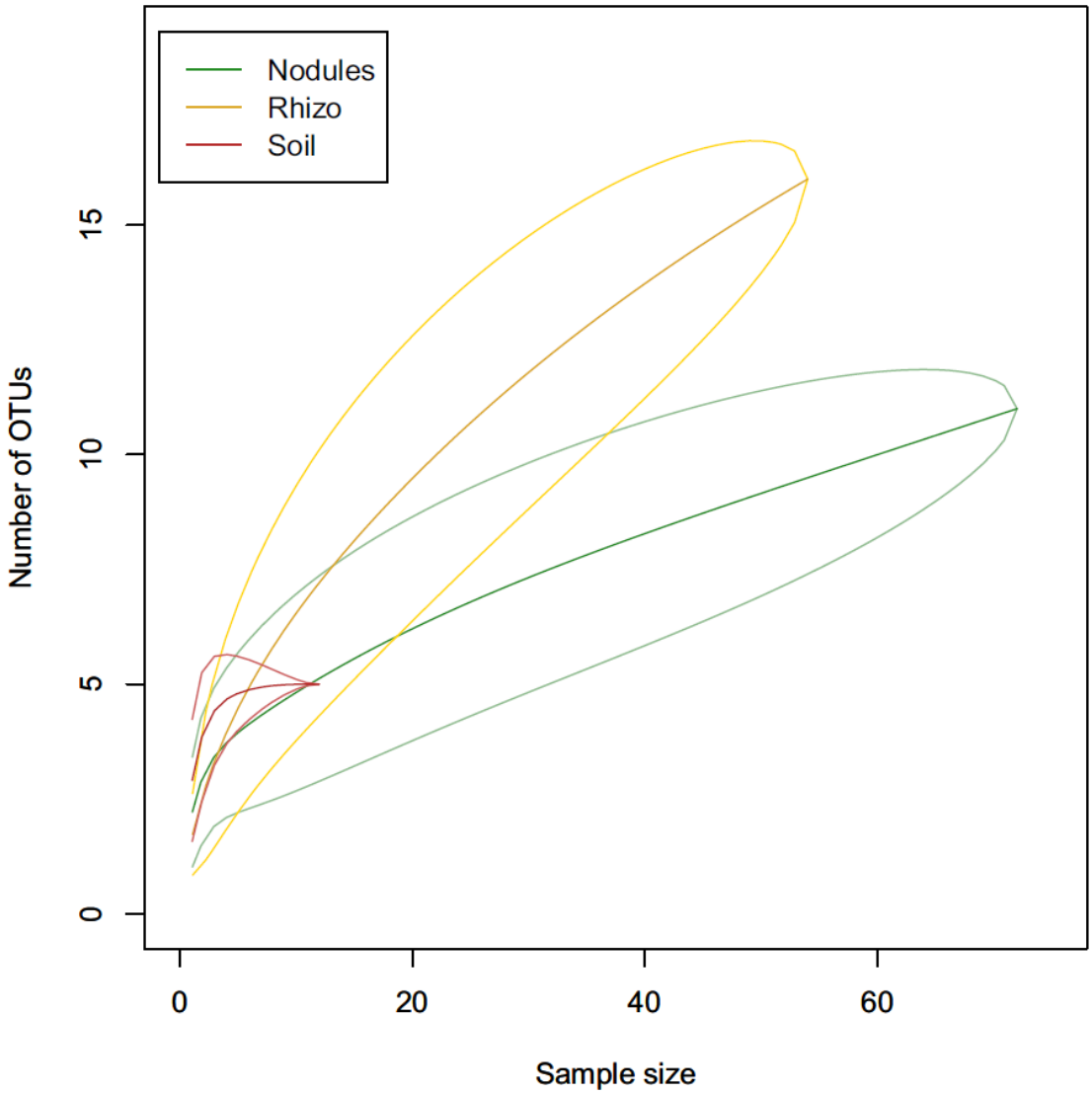

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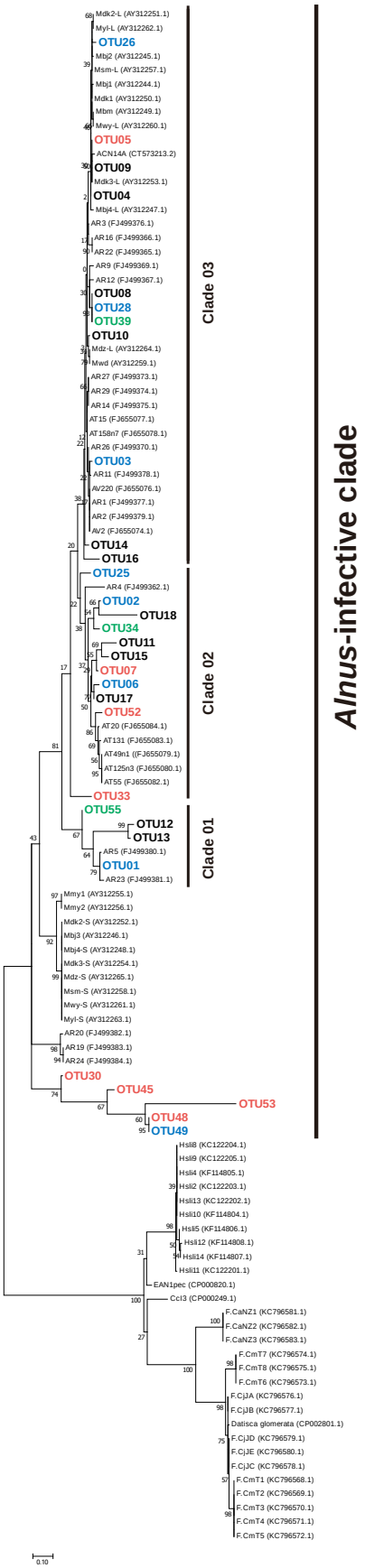
Mdk2-L (AY312251.1)
Myl-L (AY312262.1)
OTU26
Mbj2 (AY312245.1)
Msm-L (AY312257.1)
Mbj1 (AY312244.1)
Mdk1 (AY312250.1)
Mbm (AY312249.1)
Mwy-L (AY312260.1)
OTU05
ACN14A (CT573213.2)
OTU09
Mdk3-L (AY312253.1)
OTU04
Mbj4-L (AY312247.1)
AR3 (FJ499376.1)
AR16 (FJ499366.1)
AR22 (FJ499365.1)
AR9 (FJ499369.1)
AR12 (FJ499367.1)
OTU08
OTU28
OTU39
OTU10
Mdz-L (AY312264.1)
Mwd (AY312259.1)
AR27 (FJ499373.1)
AR29 (FJ499374.1)
AR14 (FJ499375.1)
AT15 (FJ655077.1)
AT158n7 (FJ655078.1)
AR26 (FJ499370.1)
OTU03
AR11 (FJ499378.1)
AV220 (FJ655076.1)
AR1 (FJ499377.1)
AR2 (FJ499379.1)
AV2 (FJ655074.1)
OTU14
OTU16
OTU25
AR4 (FJ499362.1)
OTU02
OTU18
OTU34
OTU11
OTU15
OTU07
OTU06
OTU17
OTU52
AT20 (FJ655084.1)
AT131 (FJ655083.1)
AT49n1 ((FJ655079.1)
AT125n3 (FJ655080.1)
AT55 (FJ655082.1)
OTU33
OTU55
OTU12
OTU13
AR5 (FJ499380.1)
OTU01
AR23 (FJ499381.1)
Clade 03
Clade 02
Clade 01
Mmy1 (AY312255.1)
Mmy2 (AY312256.1)
Mdk2-S (AY312252.1)
Mbj3 (AY312246.1)
Mbj4-S (AY312248.1)
Mdk3-S (AY312254.1)
Mdz-S (AY312265.1)
Msm-S (AY312258.1)
Mwy-S (AY312261.1)
Myl-S (AY312263.1)
AR20 (FJ499382.1)
AR19 (FJ499383.1)
AR24 (FJ499384.1)
OTU30
OTU45
OTU53
OTU48
OTU49
Hsli8 (KC122204.1)
Hsli9 (KC122205.1)
Hsli4 (KF114805.1)
Hsli2 (KC122203.1)
Hsli13 (KC122202.1)
Hsli10 (KF114804.1)
Hsli5 (KF114806.1)
Hsli12 (KF114808.1)
Hsli14 (KF114807.1)
Hsli1 (KC122201.1)
EAN1pec (CP000820.1)
Ccl3 (CP000249.1)
F.CaNZ1 (KC796581.1)
F.CaNZ2 (KC796582.1)
F.CaNZ3 (KC796583.1)
F.CmT7 (KC796574.1)
F.CmT8 (KC796575.1)
F.CmT6 (KC796573.1)
F.CjJA (KC796576.1)
F.CjJB (KC796577.1)
Datisca glomerata (CP002801.1)
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F.CjJE (KC796580.1)
F.CjJC (KC796578.1)
F.CmT1 (KC796568.1)
F.CmT2 (KC796569.1)
F.CmT3 (KC796570.1)
F.CmT4 (KC796571.1)
F.CmT5 (KC796572.1)
0.10
Alnus-infective clade

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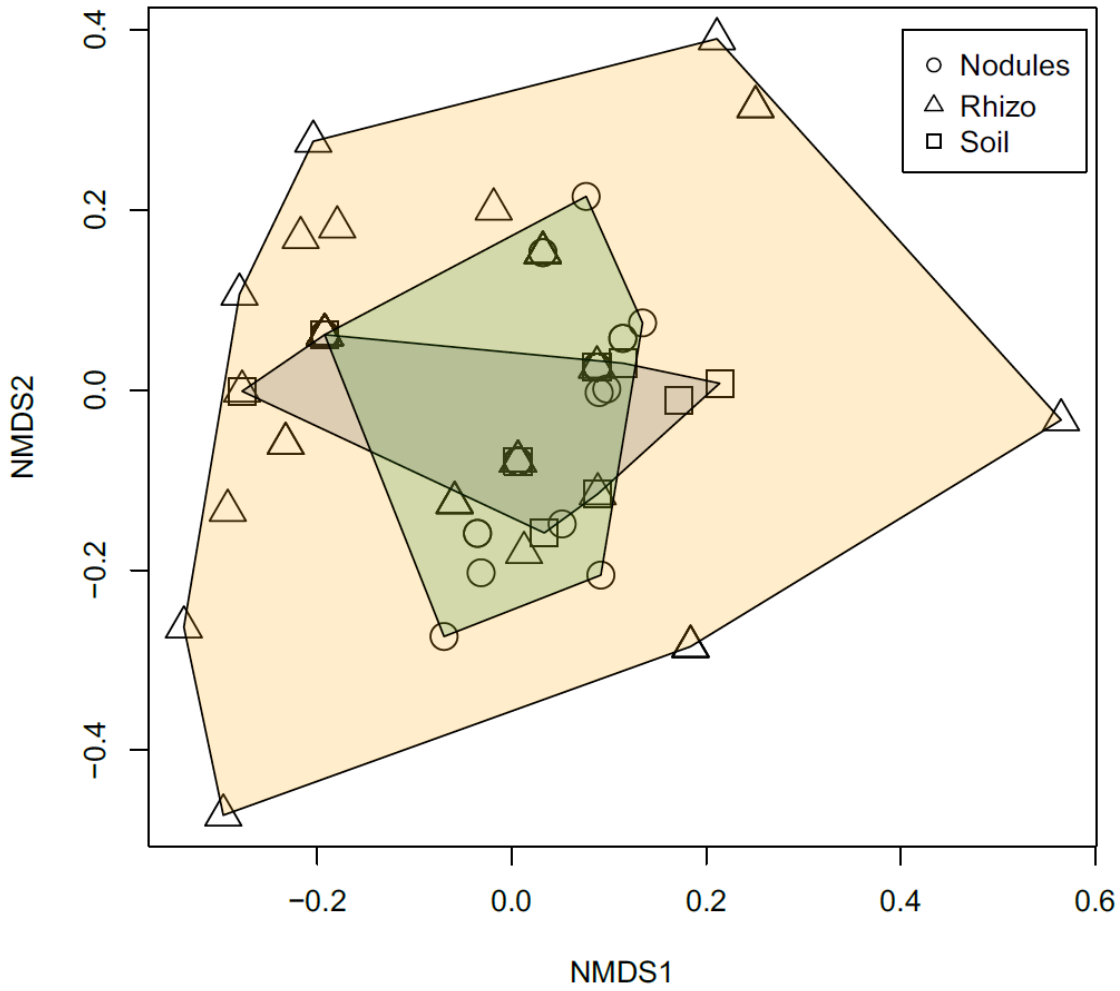

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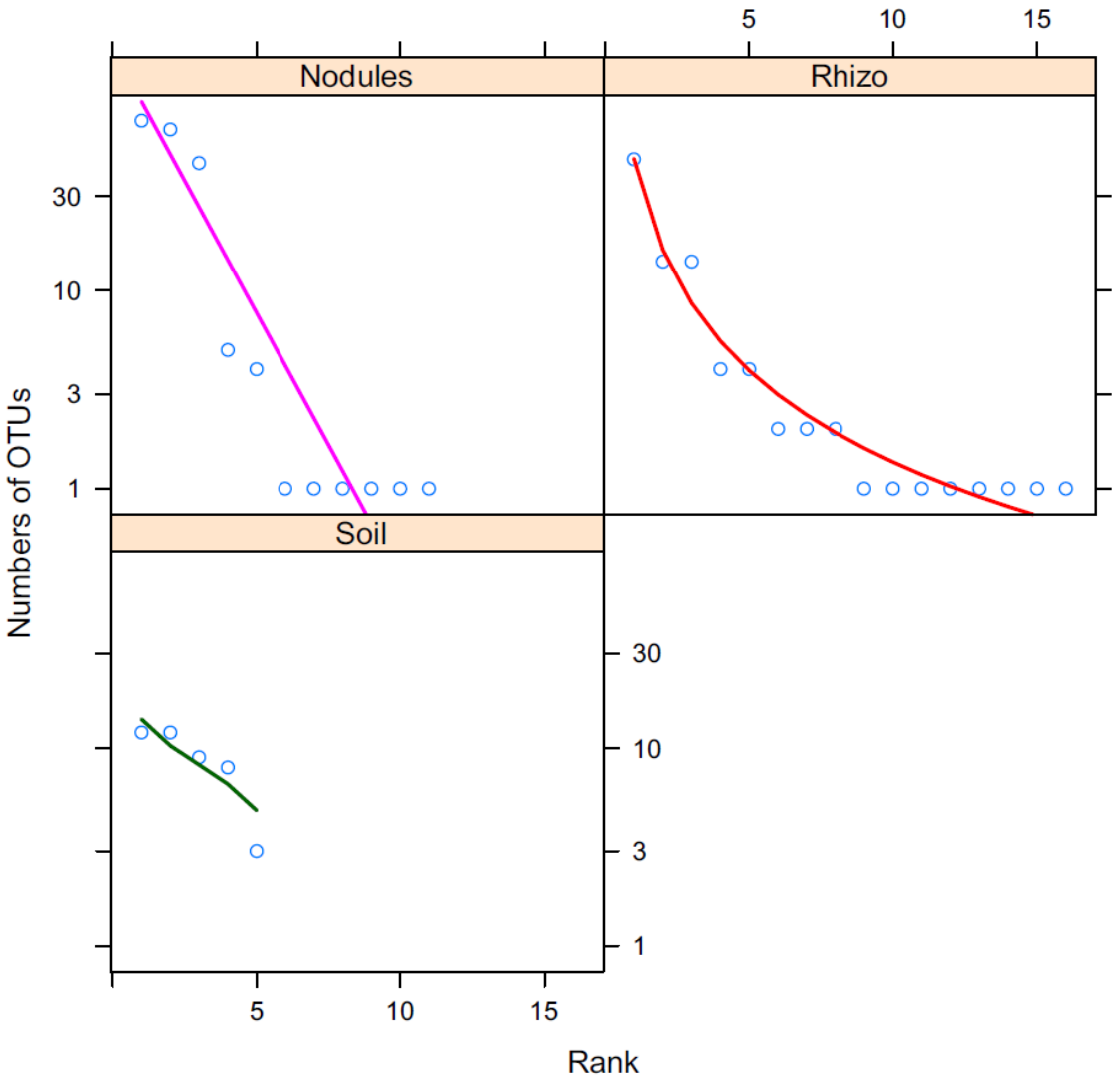

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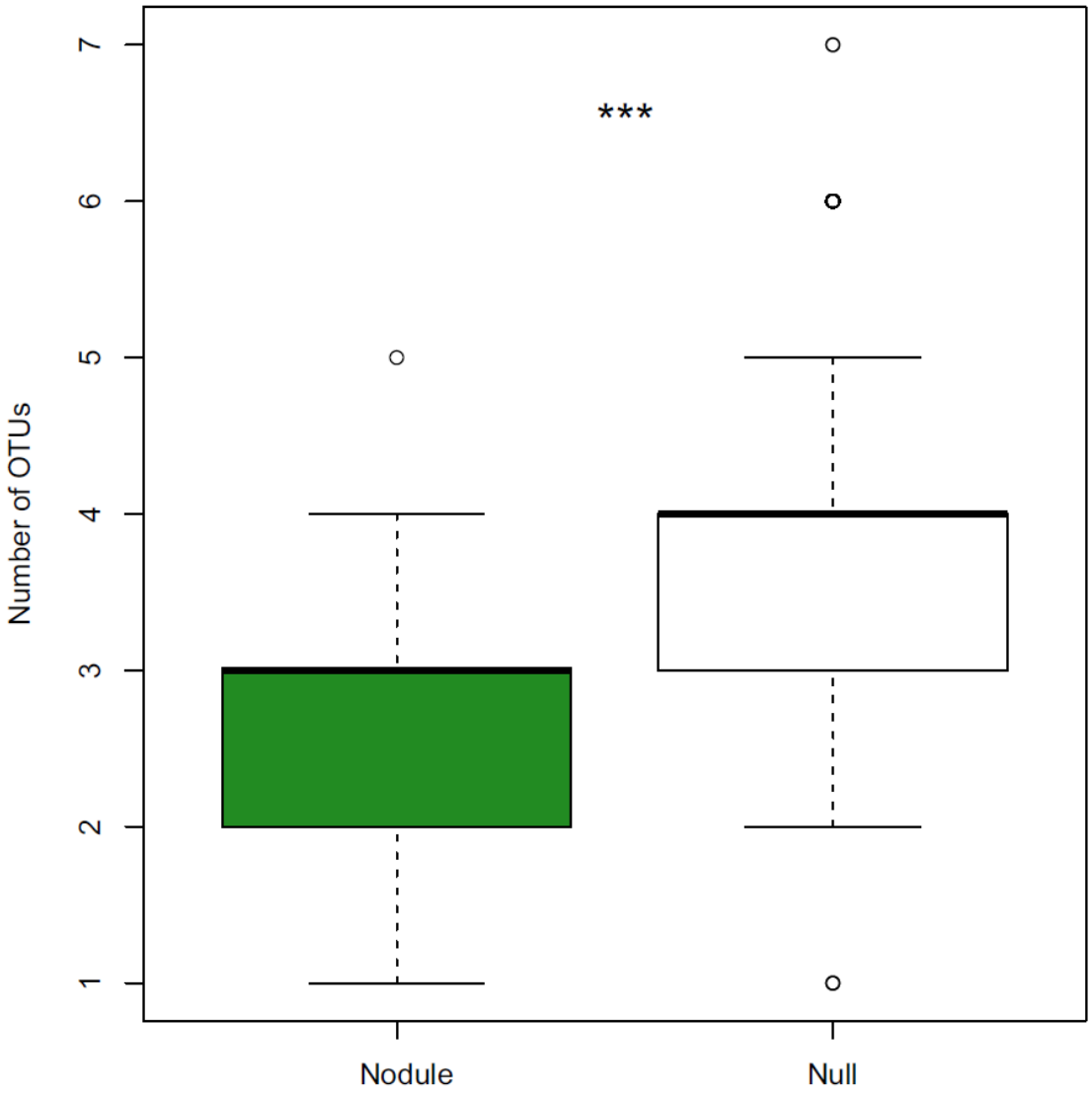

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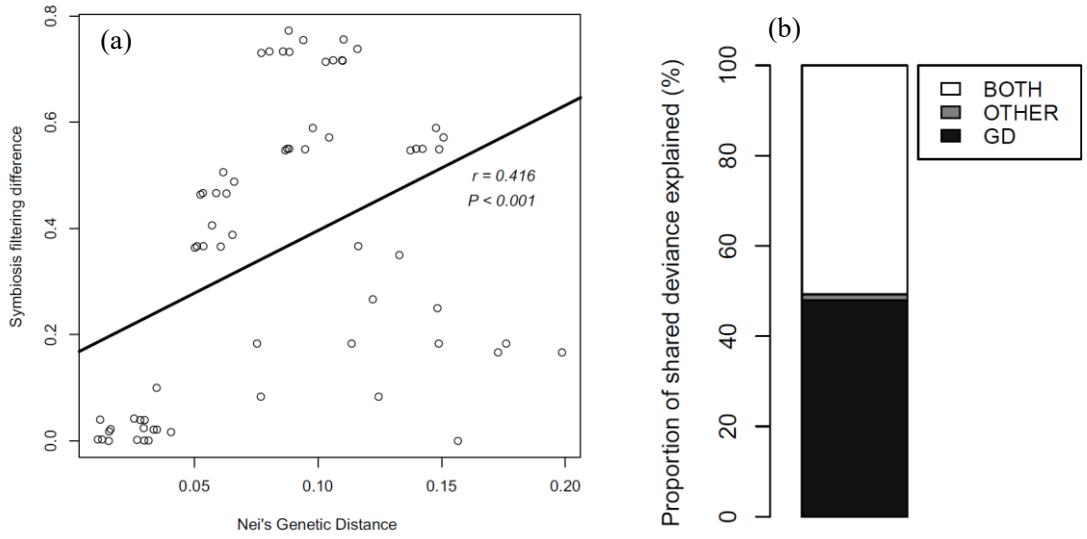

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Chapter 5. Above- and below-ground interactions between an herbivorous insect and natural symbiont community in a mature tree

Introduction

Terrestrial plants ubiquitously interact with microsymbionts such as mycorrhizal fungi and nitrogen-fixing bacteria on their roots (Fitter, 1990). These microsymbionts can have profound impacts on phenotypic variation within a plant species, including growth, survival, chemical compounds and plant defense (Daft & Nicolson, 1966; Gage, 2004; Gange & West, 1994; Gerdemann, 1968). Many studies have shown that different species of mycorrhizal fungi can influence plant–herbivore interactions (Bezemer & van Dam, 2005; Gange & West, 1994; Gehring & Bennett, 2009; Hartley & Gange, 2009; Vályi et al., 2016; Wolfe et al., 2005). For examples, infection of arbuscular mycorrhizal fungi decreases herbivory damage of a host plant (Gange & West, 1994). Similarity, in legume–rhizobium symbioses, effects of infection of nitrogen-fixing bacteria on plant–herbivore interactions have been also studied well (Ballhorn et al., 2013; Katayama et al., 2011; 2014; Kempel et al., 2009; Thamer et al., 2011; Whitaker et al., 2014). For example, Thamer et al. (2011) reported that a lima bean infected with rhizobia increased concentration of cyanogenic precursors in leaves and decreased herbivorous damages in comparison with host beans without rhizobia. Moreover, Katayama et al. (2011) demonstrated that inoculation of rhizobia indirectly affected abundance of herbivorous insects on a host plant.

However, there are two following issues that remains to be solved toward an understanding of the plant-mediated indirect effects of rhizobial symbionts on herbivores in natural ecosystems: (1) use of one host individual and one symbiotic strain, and (2) experiments with biased age of a host plant. First, a lot of previous studies manipulated infection/non-infection of a symbiont into a host individual to elucidate above- and below-ground interactions. However, the results from one host–one symbiont symbioses

are unlikely to represent consequences of above- and below-ground interactions in natural ecosystems (Gadhave & Gange, 2018). Genetic variation in rhizobial symbionts has great impacts on host phenotypes, such as growth, nitrogen contents, survival, and defense (Barrett et al. 2012; Pahua et al., 2018; Robinson et al., 2000). Furthermore, high genetic diversity of the rhizobial symbionts commonly exist in nature (Bahram et al., 2011; Mishra et al., 2015; Moawad & Schmidt, 1987; Põlme et al., 2014; Shiraishi et al., 2011). In the natural environments with high genetic diversity of rhizobial symbionts, multiple rhizobial strains coexist in a host individual (Moawad & Schmidt, 1987; Shiraishi et al., 2011; Chapter 4). Moreover, Barrett et al. (2015) showed that artificially manipulated rhizobial composition influences growth of *Acacia* hosts. Thus, realistic bacterial compositions in host plants remains to be elucidated in the studies on indirect effects between rhizobia and herbivores.

Second, the experimental studies are biased to saplings and seedlings of long-lived trees (Barrett et al., 2012; 2015; Dillon & Baker, 1982; Hooker & Wheeler, 1987; Prat, 1989; Sellstedt et al., 1986) or short-lived herbaceous plants (Ballhorn et al., 2013; Caldwell, 1966; Dean et al., 2009; 2014; Katayama et al., 2011; 2014 Miller & Sirois, 1982; Thamer et al., 2011). Although different effects between seedlings and mature plants on growth and preference of herbivorous insects have been reported (Fenner et al., 1999; Macedo & Langenheim, 1989), the knowledge whether consequences of plant–microsymbiont mutualism differ between seedlings and matures is still poor. Therefore, the studies using mature tree species, which occupy biomass in forests, are needed toward an understanding of above- and below-ground interactions in natural forests.

Alnus hirsuta–Frankia natural symbiosis model in my field provide opportunities to address above two problems because variation in *Frankia* genetic composition

interacting with mature alders under a natural condition was revealed by metabarcoding (Chapter 4). Thus, this system allows us to investigate effects of, at least, variation in *Frankia* composition among mature host alders on above- and below-ground interaction. In consistent with legume-associated rhizobia, *Frankia* also fix atmospheric nitrogen and form nodules on roots of host plants. However, *Frankia* bacteria which are gram-positive actinobacteria are phylogenetically distant from gram-negative rhizobia. In addition, most actinorhizal plants which are infected by *Frankia* are woody plants, while many legume plants are herbaceous (Wheeler et al., 2008). In particular, *Alnus* species, one of the actinorhizal plants, have the characteristics of a foundation tree species in early successional forests (Ellison et al., 2005; 2010; Kagiya et al., 2018). Thus, revealing indirect effects of the alder–*Frankia* symbiosis on growth, survival, and preference of herbivorous insects could be a crucial step toward an understanding of population and community dynamics of tree-associated arthropods in forest ecosystems.

In this study, I tested effects of the alder–*Frankia* natural symbiosis on growth, survival, and preference of a folivorous insect. For this purpose, I conducted insect feeding and preference experiments, using leaves collected from mature trees of the alder, whose associated *Frankia* composition were revealed (Chapter 4). The natural symbiosis model allows us to use the realistic compositions of mutualistic partners and mature trees. Specifically, the goal of this study is to test the following three hypotheses. First, the amount of carbon-based defensive chemicals of the alder differ among the bacterial compositions. Rhizobia infection consumes carbon resources of host plants (Chapin et al., 1987; Finke et al., 1982; Kaschuk et al., 2009), and in actinorhizal symbiosis, decrease in carbon-based leaf traits by *Frankia* infection increased folivorous damages in *Alnus* seedlings (Ballhorn et al., 2017). Because the carbon costs of nodulation are altered by

genetic variation in bacterial symbionts (Kaschuk et al., 2012; Skøt et al., 1986), the costs may be differed among bacterial compositions. Second, the realistic compositional variation in bacterial symbionts influence growth, survival and preference of a folivorous insect. Finally, each member in the bacterial composition has a distinct impact on performance of a folivorous insect.

Materials and Methods

Organisms

Alnus hirsuta Spach (Betulaceae) is a deciduous broadleaf tree and an early successional species. It is widely distributed in temperate riparian forests in Japan, northeastern China, Korea, and Russia. *Alnus* genus plants mutualistic interacts with nitrogen-fixing *Frankia* actinobacteria (*Frankia* sp.: Frankiaceae).

The leaf beetle (*Agelastica coerulea* Baly: Chrysomelidae) is an oligophagous folivorous insects feeding on alders. It is commonly distributed in Japan, northeastern China, Korea, and Russia. Adult beetles overwinter under a surface part of soil and occur from late April to early June in Hokkaido, Japan. The beetles oviposit eggs from late May to June. Larvae hatch after c. 10 days from egg oviposition. Hatched larvae immediately begin to feed on leaves of alders. The larvae start to pupate after c. 20 days from hatching and complete pupation after c. 10 days from the initiation of pupation (Sakikawa et al., 2016).

Field collection of insects and leaves

Twenty-two adults in total of the leaf beetle were collected from an adult tree of *A. hirsuta*

in Nayoro (N 44° 19’ 39.8”, E 142° 28’ 36.9”), Hokkaido, Japan, in late May 2019. Collected beetles were confined in a plastic case to gain their eggs. When female beetles started to oviposit, each female was separated into each petri dish (⌀ 12 cm). 180 larvae in total were obtained from four females for subsequent feeding experiments. After I gained these larvae, all adult beetles were separated into each petri dish (⌀ 12 cm) in an environmental chamber at 23 °C and 12 h light cycle/day. Leaves of the alder individual which the beetles were collected from were provided every three days.

For subsequent experiments, I collected leaves of 15 alder individuals from three subpopulations, which have different *Frankia* composition (Table 5-1), in Uryu Experimental Forest of Hokkaido University. The leaves were collected in late May and mid-June 2019. The collected leaves were immediately stored in refrigerator at 5 °C.

Chemical analysis

To analyze carbon-based leaf traits, one of the collected leaves was dried in an oven at 38 °C for one week. Dried leaves were ground using a mixer mill. To extract phenolics and tannin in the leaves, 25 mg of the grinded leaf samples was shaken with 12 ml 50% (v/v) methanol in polypropylene test tube at 70° for an hour. The extracted solution was centrifuged at 12,000 × g for 10 minutes. Chemical contents of total phenolics and condensed tannin in the supernatant were analyzed by the Folin–Ciocalteu method and the butanol–hydrochloric acid method (Tahvanainen et al., 1985), respectively.

Feeding experiment

The 180 larvae on fed leaves of the 15 different alders immediately after hatching in petri dishes (⌀ 9 cm). Each larva was confined in a petri dish in an environmental chamber at

23 °C and 12 h light cycle/day. After 20 days, I measured weight of larval individuals. When I completed measurement of the weight, I laid commercial leaf mold on half depth of each the petri dish for pupation of the larva. After 30 days from hatching, individuals with successful pupation were recorded as surviving ones.

Preference test

Twenty-two adult beetles, which were collected in Nayoro as described above, were used for a choice experiment. I prepared a choice arena of a Petri dish (⌀ 12 cm) with a pair of two leaf disks (⌀ 11.5 mm), one of which was selected from the 15 alder individuals (Table 5-1), and the other was selected from one of the rest alders. Each pair of leaf disks were placed on the edges of a petri dish in 12 cm distance. One of the collected adult beetles was put on the center of a Petri dish. I recorded the leaf that was firstly bitten by the beetle (but visitation without bites was not recorded). I performed the choice assay with all combinations of possible pairs with three replications (${}_{15}C_2 \times 3$ reps = 315 trials in total).

Statistical analysis

Effects of phenolics and tannin contents in leaves on growth, survival and preference of the leaf beetle

Effects of phenolics and tannin contents in the leaves on growth, survival and preference of the beetle larvae were examined using generalized linear mixed model (GLMM). The three following models with either total phenolics and tannin contents as fixed factors were performed: (1) including larval growth as response factors with log-linked gamma distribution, and host individuals and maternal identities of larvae as random factors, and

(2) including larval survival as response factors with a binomial distribution, and host individuals and maternal identities of larvae as random factors.

To test effects of foliar chemical traits on preference of the adult beetles, the models with binomial distribution included pairwise differences of either phenolics and tannin contents as fixed factors, and pairwise combinations of host individuals as random factors. To illustrate effects of the leaf traits on preference of the beetles, I evaluated preference indices in Lhomme et al. (2018). Firstly, I categorized the values of pairwise differences of the foliar chemical traits as the “High” category and the “Low” category because the data has to be discrete values to calculate the preference indices. The High and Low categories represented a higher part and a lower part of tertile of the pairwise difference data of chemical traits, respectively. I calculated the preference indices based on these categories as follows:

$$\text{Preference index of the leaf traits} = \frac{(\text{"High"}\ choice - \text{"Low"}\ choice)}{total\ choice}.$$

Effects of symbiont composition on total phenolics and condensed tannin contents in leaves

Leaf traits (i.e., total phenolics and tannin contents; n = 60) as response variables were analyzed by GLMM with a log-linked gamma distribution. Effects of *Frankia* composition on leaf traits were analyzed using the following models, including *Frankia* composition (Table 5-2) as fixed factors, and *Alnus* individuals and subpopulations as random factors. Effects of each *Frankia* member on the leaf traits were analyzed the model which included the presence or absence of OTU02, OTU03 and OTU25 as fixed factors, and host individuals and subpopulations as random factors.

Effects of symbiont composition on growth, survival and preference of the herbivore

Growth (n = 162) and survival (n = 179) of the larvae as response variables were analyzed, using GLMM with a log-linked gamma distribution and binomial distribution, respectively. To examine effects of *Frankia* composition on these response variables, I modeled the dataset including *Frankia* composition as fixed factors, and maternal identities of larvae and alder individuals and subpopulations as random factors. Pairwise comparisons were performed for the results of these models using Tukey's HSD tests with the R package multcomp 1.4-10.

In order to examine effects of *Frankia* composition on preference of the adult beetles, the choice of larvae for either *Frankia* composition (n = 630) was modeled, using GLMM with a binomial distribution. Effects of *Frankia* composition were tested, using the models including pairwise combinations of *Frankia* composition as fixed factors (i.e., interactions of *Frankia* composition between focal leaf and pair leaf, except for the same combinations), and pairwise combinations of host individuals (i.e., interactions of alder individuals between focal leaf and pair leaf, except for the same combinations) and subpopulations (i.e., interactions of alder subpopulations between focal leaf and pair leaf, except for the same combinations) as random factors. To illustrate effects of *Frankia* composition on preference of the beetles, I evaluated preference indices (Lhomme et al., 2018) as the following equation:

$$\text{Preference index} = \frac{(focal\ leaf\ choice - paired\ leaf\ choice)}{total\ choice}.$$

Effects of each Frankia strain on growth, survival and preference of the beetle

Effects of each *Frankia* member on the response factors were analyzed, using a model, which included the presence or absence of OTU02, OTU03 and OTU25 strains as fixed factors and host subpopulations, maternal identities of larvae, and host individuals as random factors. Effects of each *Frankia* member on the preference of beetles were examined, using a model, which included pairwise combinations of each of OTU02, OTU03 and OTU25 as fixed factors (i.e., interactions of *Frankia* strains between focal leaf and pair leaf, except for the same combinations), and pairwise combinations of host individuals and subpopulations as random factors.

All the above analyses were performed using with the lme4 1.1-21 package of the R 3.6.1 software.

Results

Phenolics and tannin contents in host leaves

Phenolics and tannin contents in alder leaves did not significantly affect both growth (Table 5-2; total phenolics: $\chi^2 = 0.382$, $P = 0.537$; tannin: $\chi^2 = 0.220$, $P = 0.639$) and survival (Table 5-2; total phenolics: $\chi^2 = 0.267$, $P = 0.605$; tannin: $\chi^2 = 0.319$, $P = 0.572$) of beetle larvae. However, I found that adult beetles significantly preferred leaves with lower tannin content (Fig. 5-1, Table 5-2; total phenolics: $\chi^2 = 3.068$, $P = 0.080$; tannin: $\chi^2 = 5.736$, $P < 0.05$). This indicated that intraspecific variation in alders in the forest would affect preference of the leaf beetle adult.

On the other hand, differences in *Frankia* composition did not significantly influence total phenolics (Fig. 5-2a; $\chi^2 = 3.695$, $P = 0.449$) and tannin contents (Fig. 5-2b; $\chi^2 = 1.727$, $P = 0.786$) in the alder leaves.

Growth, survival and preference of the leaf beetle among *Frankia* compositions

As well as foliar phenolics and tannin contents, differences in *Frankia* composition did not significantly associate with larval growth (Fig. 5-3a; χ^2 = 1.508, P = 0.825) and preference of the leaf beetle (Fig. 5-3c; χ^2 = 22.634, P = 0.307). However, I detected significant effects of *Frankia* composition on survival of the larval beetle (Fig. 5-3b; χ^2 = 9.846, $P < 0.05$). Survival of the larva differed 1.22–3.25 folds among the *Frankia* compositions.

To elucidate effects of each *Frankia* strain on growth, survival, and preference of the leaf beetle, I examined effects of the presence/absence of each strain on the leaf beetle. For larval survival of the leaf beetle, the presence of OTU02 significantly improved survival of the larvae 1.67-fold than the absence of OTU02 (Fig. 5-4b; χ^2 = 4.340, $P <$ 0.05). In contrast, the presence of OTU03 strain significantly decreased survival 0.45-fold than the absence of OTU03 (Fig. 5-4b; χ^2 = 5.541, $P < 0.05$). There were no significant differences in larval growth between presence and absence of each of OTUs (Fig. 5-4a; OTU02: χ^2 = 0.236, P = 0.627; OTU03 χ^2 = 0.778, P = 0.378; OTU25: χ^2 = 0.025, P = 0.875) and preference (Fig. 5-4c; OTU02: χ^2 = 4.977, P = 0.083; OTU03: χ^2 = 0.656, P = 0.720; OTU25: χ^2 = 1.420, P = 0.492) The presence/absence of OTU25 did not affect both growth and survival of the larvae (Fig. 5-4b; survival: χ^2 = 0.439, P = 0.507). These findings suggested that these *Frankia* strains indirectly affected survival of the larval beetle, mediated through altering plant traits other than total phenolics and tannin contents.

In addition, preference of adult beetles was significantly affected by alder subpopulations (Fig. 5-5c; χ^2 = 18.159, $P < 0.01$) while survival of beetle larvae was not significantly influenced by host subpopulations (Fig. 5-5c; χ^2 = 0.168, P = 0.920). This

result suggests that effects of *Frankia* composition on larval survival rate are unlikely to be resulted from differences of abiotic factors such as climate and soil conditions, and genetic identities of the alder among host subpopulations.

Discussion

This study demonstrated that belowground *Frankia* genetic composition had a profound impact for the survival rate of the above-ground leaf beetle. The difference in *Frankia* composition did not associate with foliar tannin content, which significantly affected preference of the leaf beetle. However, *Frankia* composition influenced the survival rate of the larvae of the leaf beetle. The effect of bacterial composition on larval survival was likely to be resulted from specific effects of each of bacterial strains.

Costs of mixed infection of *Frankia*

In general, it is known that host plants lose carbon resources to supply them for bacterial symbionts (Chapin et al., 1987; Finke et al., 1982; Kaschuk et al., 2009). For examples, Finke et al. (1982) reported that soybeans with rhizobia lost approximately 25% of photosynthetic carbon resources to invest into root nodules in comparison with soybeans without rhizobia. In actinorhizal symbiosis, Ballhorn et al. (2017) showed that *Alnus* seedlings with *Frankia* bacteria had lower amount of carbon-based compounds such as carbohydrates and tannin than *Frankia*-free seedlings. However, the present study demonstrated that composition of bacterial symbionts did not affect the amount of carbon-based leaf compounds in a host plant (Fig. 5-2). There are three possible explanations for the contrasting result. First, nodulation costs may differ among host life stages such as

matures and seedlings. Relative costs of symbiosis formation may be less for mature trees than seedlings due to differences in total photosynthetic production and carbon storage (Kaschuk et al., 2009). Second, the sum of carbon supply for successfully infecting symbiont communities in the field may be constant, regardless of *Frankia* genetic composition. Third, genetic variation in carbon-based defensive traits in host plant may mask symbiont community effects. These explanations are mutually exclusive. Future studies should pay more attention to nodulation costs over life stages of host plants and alteration in defensive traits of hosts by genetic variation in bacterial symbionts toward an understanding of above- and below-ground interactions in nature.

The role of genetic variation in *Frankia* strains on above- and below-ground interactions

Frankia composition significantly altered the survival of the larvae (Fig. 5-3b). The survival in this study included pupation rate, and the contribution of the pupation rate to the survival was greater (94/179 larval individuals) than the mortality during larval stage before pupation initiation (16/179 larval individuals). Thus, *Frankia* composition could affect the pupation rate of herbivorous larvae. A few studies reported effects of plant allelochemical compounds on the pupation of herbivorous insects. For example, Zebelo et al. (2016) demonstrated that inoculation of growth-promoting rhizobacteria to a cotton plant increased terpenoid concentration and significantly reduced pupation rate of beet armyworm. Also, Ramachandran et al. (1998) showed that one *Brassica* strain with high resistance tended to reduce pupation rate of diamondback moth without decrease of larval mass. Low survival at pupal stage may be due to peroxidase or other enzymes and toxins (Kruess, 2002). Plants might develop it as one of defense strategies against herbivory,

which reduce the successful pupation of larvae to inhibit future fecundity of herbivores (Shelton, 2004).

Effects of *Frankia* compositions on survival of herbivores were likely to be resulted from different functions of *Frankia* strains because herbivore responses were inconsistent between OTU02 and OTU03 strains (Fig. 5-4b). The two following functions may underlie the different role among *Frankia* strains. First, the host plant might receive different nitrogenous compounds among *Frankia* strains. Fixed nitrogen from bacterial symbionts is stored in host plants as specific forms such as ureides and amides (Berry et al., 2011; Dean et al., 2009; Schubert, 1986). Allantoin, a type of ureides, in a legume plant reduced growth and survival of an herbivorous insect (Wilson & Stinner, 1984). In fact, the nitrogen from rhizobia is more useful to produce legume plant defense against herbivory than fertilized nitrogen (Dean et al., 2014). Although whether the specific forms of nitrogen resource in plant storage depend on genetic variation in bacterial symbionts has not been known yet, genetic variation in nitrogen form from *Frankia* strains could partially explain the effects on survival of the herbivorous insect if specific nitrogen form depend on genetic basis of nitrogen-fixing bacteria.

Second, the growth promotion of host plants is driven by several *Frankia* functions such as phytohormone synthesis and enzyme production other than nitrogenase (Glick, 1995; 1998; Hirsch et al., 1997; Péret et al., 2007; Perrine-Walker et al., 2010; Sayed et al., 2002; Tisa et al., 2016). Recently, Nouioui et al. (2019) discovered genetic basis of some *Frankia* functions that associated with plant growth, e.g., genes which encode for products associated with the biosynthesis and for lytic enzymes and chitinases. Genetic variation between *Frankia* OTU strains might represent the differences in these *Frankia* functions.

Field approach for consequences of above- and below-ground interactions

Traditional studies have addressed indirect effects of rhizobial symbionts for herbivores via a host plant, artificially manipulating partnerships in plants–bacteria symbioses (Ballhorn et al., 2013; Katayama et al., 2011; 2014; Kempel et al., 2009; Thamer et al., 2011; Whitaker et al., 2014). Thus, previous studies had limitations for an understanding of above- and below-ground interactions in natural ecosystems. My study at least partially overcome the limitation, using a system of mature trees and known symbiont communities in the field. In addition, genetic variation in a host alder and environmental variation across focal host subpopulations may not be large because the subpopulations were genetically closed based on numerous SNPs using RAD-seq (Chapter 2) and pH of soils, at least, were not significantly differ among the subpopulations (linear model: χ^2 = 4.334, P = 0.115). My field approach has a potential to archive a breakthrough for the limitation in manipulation experiments.

Because symbiotic composition was obtained from the metabarcoding of limited number of nodules (Chapter 4), I might not completely discover all OTUs in symbiotic composition. However, analyses in Chapter 4 revealed that the *Frankia* composition was constructed by a highly limited range of strains than expected by chance. Therefore, impacts of variation in *Frankia* composition among host individuals on a folivorous insect are likely to be relevant.

This study, for the first time, demonstrated that the natural association between a host plant and bacterial symbionts influenced survival rate of an herbivore. Furthermore, alder subpopulations affected preference of the leaf beetle (Fig. 5-5c). These results suggested that natural *Frankia* compositions and *Alnus* genetic identities may affect

different components of preference and performance of herbivorous insects in the field mutualistic system because *Frankia* compositions and genetic distance of the alder did not correlate (Chapter 4). In other word, genetic variation in host plants and bacterial symbionts could separately influence different assembly processes of arthropods, such as dispersal (due to preferential behavior) and biotic filtering (due to survival performance). Future studies should pay attention to the relative importance of genetic variation in host plants and microsymbionts on shaping associated community structure in natural ecosystems.

Tables

Table 5-1. The list of the presence/absence of *Frankia* OTUs in alders.

Alnus ID	Sub population	OTU01	OTU02	OTU03	OTU25
014	BT1	**Presence**	**Presence**	Absence	Absence
021	BT1	**Presence**	**Presence**	**Presence**	Absence
024	BT1	**Presence**	**Presence**	Absence	Absence
026	BT1	**Presence**	**Presence**	**Presence**	Absence
029	BT3	**Presence**	**Presence**	Absence	Absence
031	BT3	**Presence**	**Presence**	Absence	Absence
033	BT3	**Presence**	Absence	Absence	Absence
038	BT3	**Presence**	Absence	Absence	Absence
040	BT3	**Presence**	**Presence**	Absence	**Presence**
047	BT4	**Presence**	**Presence**	Absence	**Presence**
048	BT4	**Presence**	**Presence**	Absence	**Presence**
052	BT4	**Presence**	Absence	**Presence**	Absence
053	BT4	**Presence**	Absence	Absence	Absence
054	BT4	**Presence**	Absence	Absence	Absence
055	BT4	**Presence**	**Presence**	Absence	Absence

Table 5-2. The GLMM results of the effects of carbon-based leaf traits on the performances of the leaf beetles. The χ^2 values are shown. A bold value indicates statistically significance.

	Growth	Survival	Preference
Phenol	0.3818	0.2672	3.0682
Tannin	0.2196	0.3188	**5.7364***

*: $P < 0.05$

Figure legends

Fig. 5-1. Effects of total phenolics or condensed tannin in leaves on preference of the adult leaf beetle. An asterisk indicates $P < 0.05$.

Fig. 5-2. The leaf traits among *Frankia* composition and each OTU strain. These figures indicate amounts of total polyphenolics (a) and tannin contents (b). Different colors in the figure represent the different members in the compositions i.e., OTU02 (blue), OTU03 (yellow), and OTU25 (red). White color means the composition with only OTU01. Mixed colors indicate that the composition is constructed by the members represented by the colors. Error bars show SEs.

Fig. 5-3. Effects of *Frankia* composition on growth, survival and preference of the leaf beetle. Different colors represent the different members in the compositions, i.e., OTU02 (blue), OTU03 (yellow), and OTU25 (red). Mixed colors represent that the composition is constructed by the members represented by the colors. White color means the composition with only OTU01. Error bars show SEs. Different letters represent significant differences ($P < 0.05$) using Tukey's HSD tests.

Fig. 5-4. Growth, survival and preference of the leaf beetle between the presence or absence of each OTU strain. Blue color represents presence of OTU02. Yellow color represents presence of OTU03. Red color represents presence of OTU25. White color represents absence of each OTUs. Error bars show SEs. An asterisk indicates $P < 0.05$

Fig. 5-5. Effects of alder subpopulations on growth (a), survival (b) and preference (c) of the leaf beetle. Error bars show SEs. Different letters represent significant differences ($P < 0.05$), using Tukey's HSD tests.

Figures

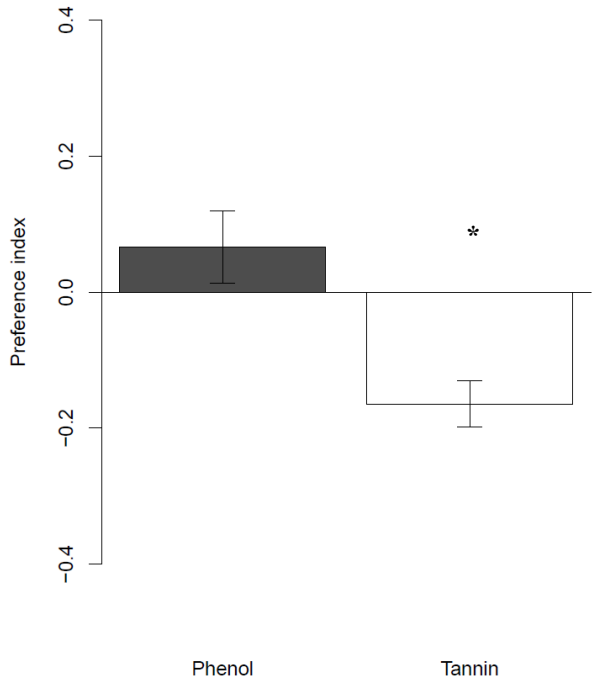

Fig. 5-1. Effects of total phenolics or condensed tannin in leaves on preference of the adult leaf beetle. An asterisk indicates $P < 0.05$.

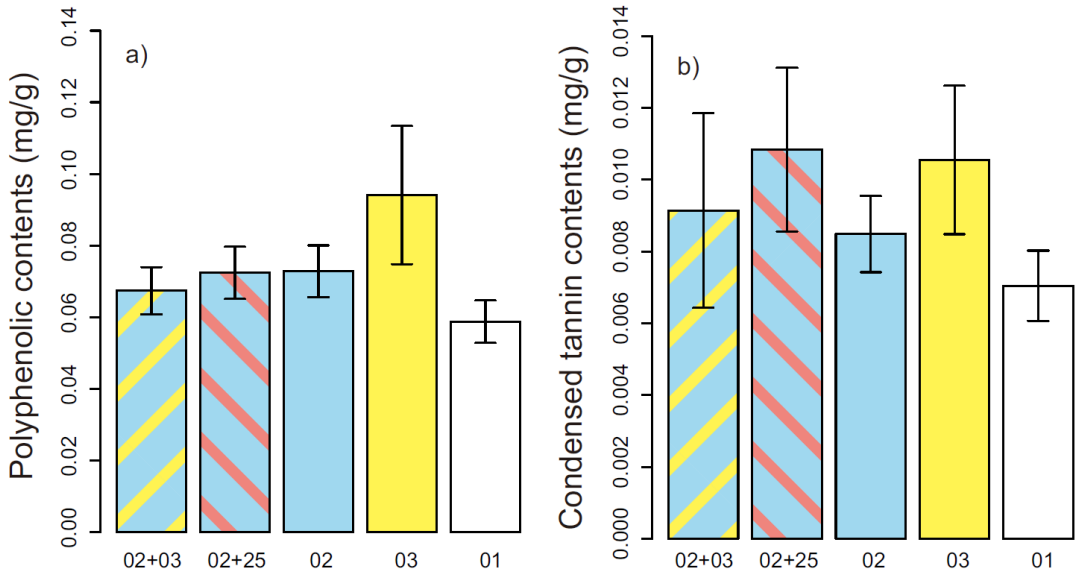

Fig. 5-2. The leaf traits among *Frankia* composition and each OTU strain. These figures indicate amounts of total polyphenolics (a) and tannin contents (b). Different colors in the figure represent the different members in the compositions i.e., OTU02 (blue), OTU03 (yellow), and OTU25 (red). White color means the composition with only OTU01. Mixed colors indicate that the composition is constructed by the members represented by the colors. Error bars show SEs.

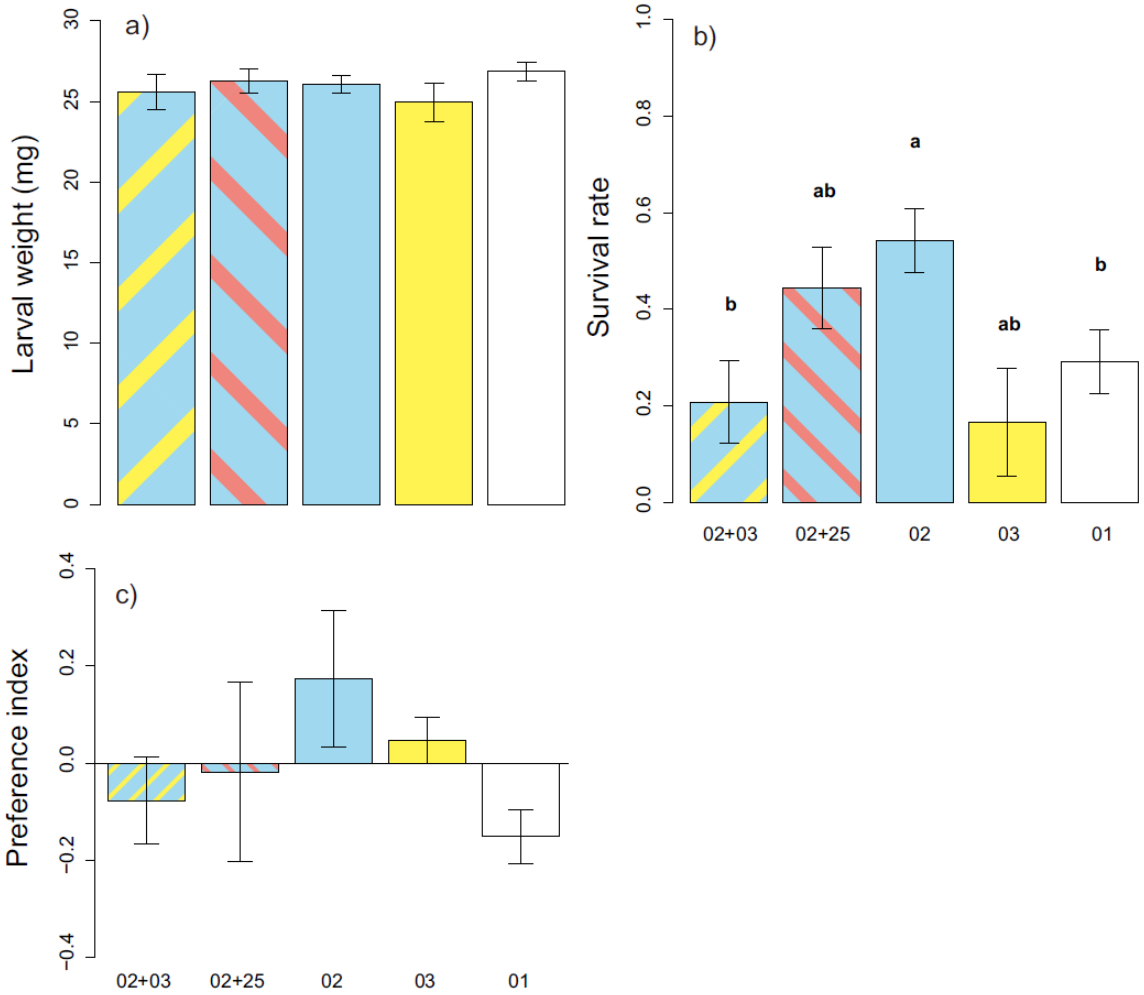

Fig. 5-3. Effects of *Frankia* composition on growth, survival and preference of the leaf beetle. Different colors represent the different members in the compositions, i.e., OTU02 (blue), OTU03 (yellow), and OTU25 (red). Mixed colors represent that the composition is constructed by the members represented by the colors. White color means the composition with only OTU01. Error bars show SEs. Different letters represent significant differences ($P < 0.05$) using Tukey's HSD tests.

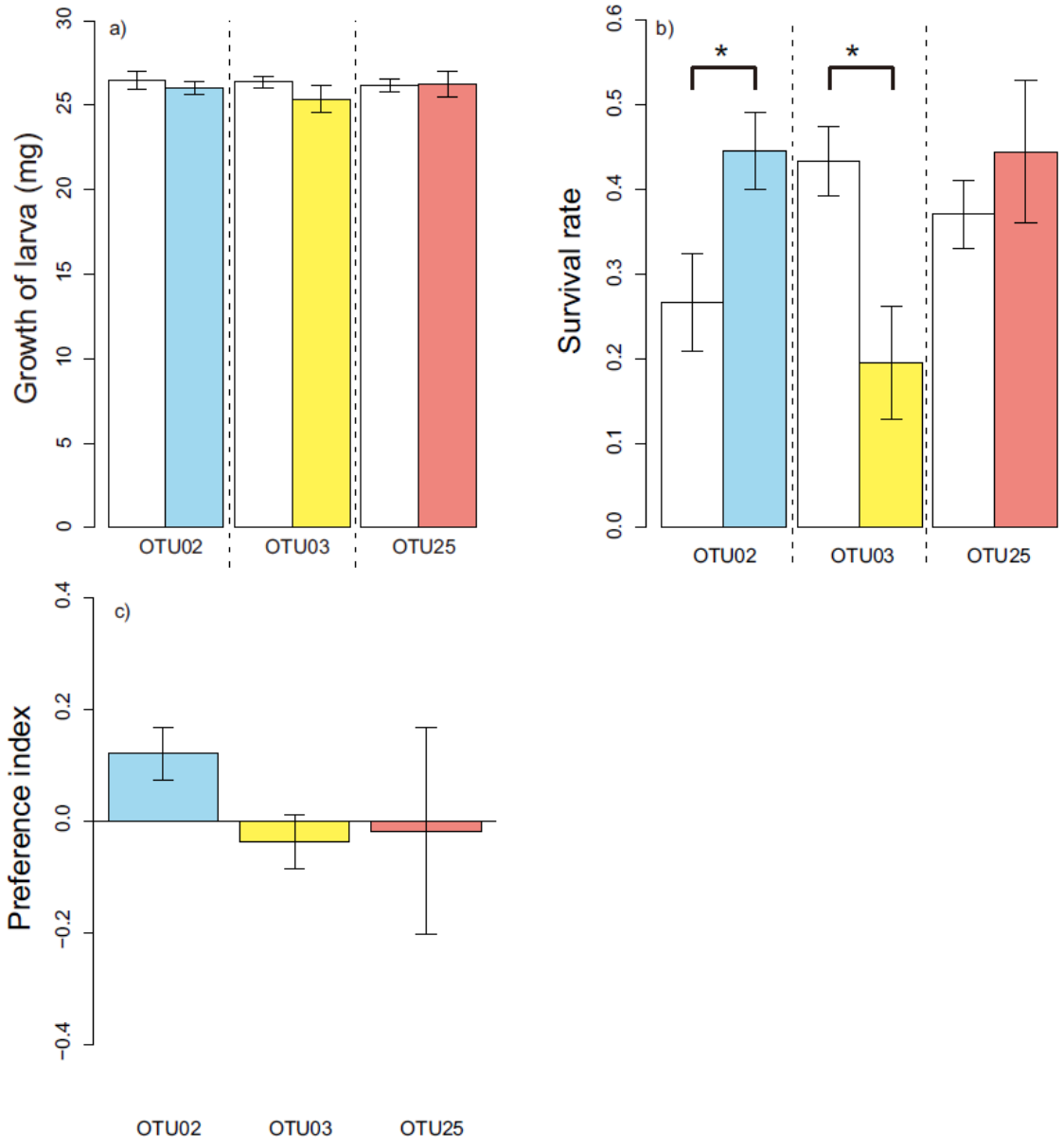

Fig. 5-4. Growth, survival and preference of the leaf beetle between the presence or absence of each OTU strain. Blue color represents presence of OTU02. Yellow color represents presence of OTU03. Red color represents presence of OTU25. White color represents absence of each OTUs. Error bars show SEs. An asterisk indicates $P < 0.05$

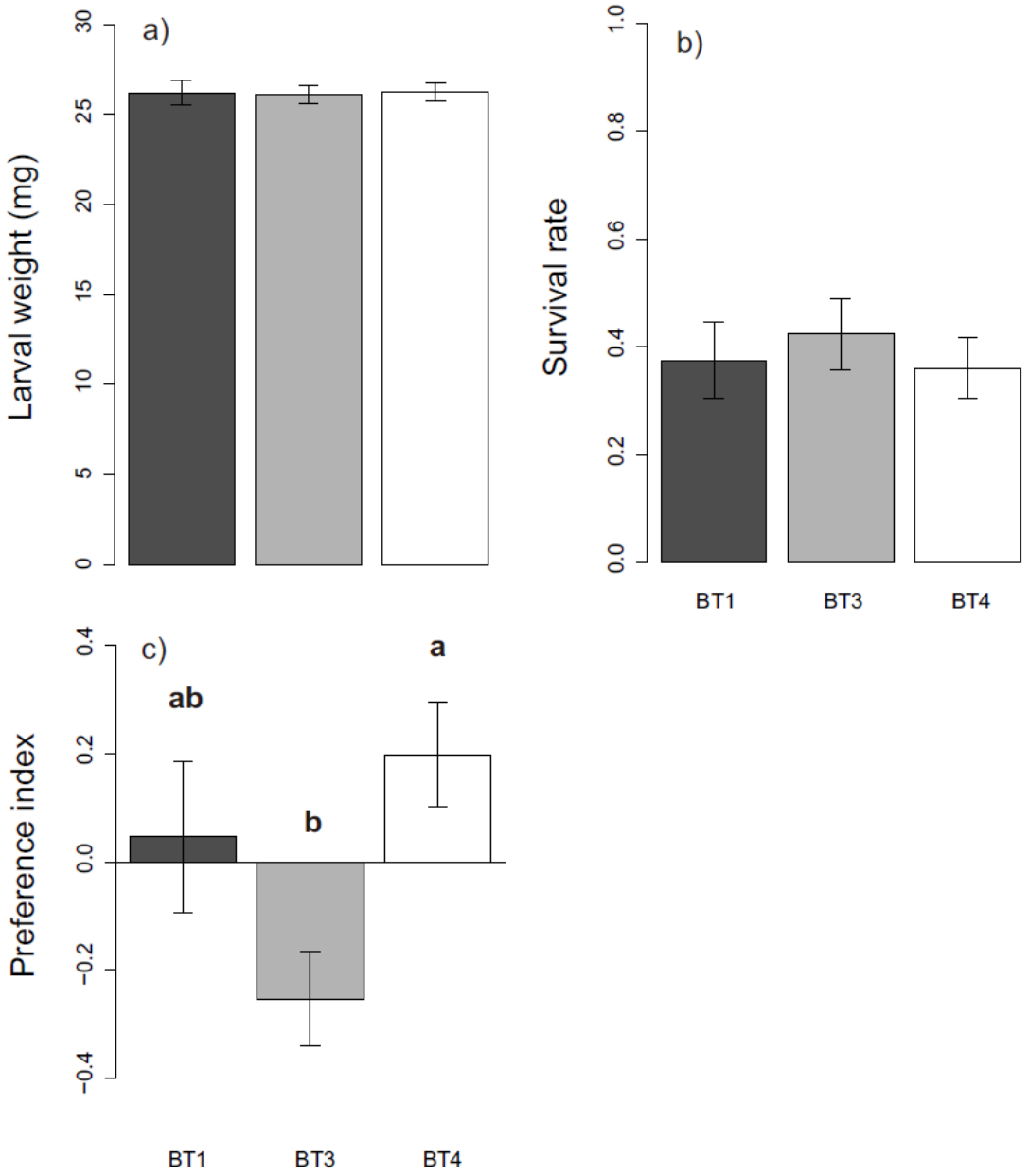

Fig. 5-5. Effects of alder subpopulations on growth (a), survival (b) and preference (c) of the leaf beetle. Error bars show SEs. Different letters represent significant differences ($P < 0.05$), using Tukey's HSD tests.

Chapter 6. General discussion

This thesis clearly demonstrated the importance of genetic variation in a foundation species on assembly processes of both above- and below-ground communities in the field. In this chapter, I summarize the results obtained in this study, and discuss the integration of mutualism into the framework of community genetics.

In Chapter 2, I investigated whether genomic variation in a foundation tree species predicts community structure of associated arthropod, using numerous genetic markers of alders from genome-wide analysis with RAD-seq. As a result, natural genomic variation in an alder species strongly predicted community structures of associated arthropods in a forest. Moreover, genetic variation in an alder was the most important predictor of other measured factors in this study. These findings support genetic similarity rules (Bangert et al., 2006) in a natural ecosystem.

In Chapter 3, to reveal genetic structure of infective-*Frankia* in a forest, *Frankia* DNA in more than 200 root-nodules from alder seedlings were analyzed. As a result, 18 *Frankia* genotypes were distributed as mosaic-like genetic structure in the field.

In Chapter 4, to test the hypothesis that a host plant interacts with specific strains of mutualistic bacteria even in a field with high genetic diversity of the mutualistic bacteria, I comprehensively analyzed genetic community of *Frankia* in host individuals and in surrounding soils using metabarcoding with NGS. As a result, while most of alder individuals interacted with multiple genotypes of *Frankia* bacteria, the *Frankia* composition interacted with host alders was constructed by consistent members of *Frankia* despite of high genetic diversity of *Frankia* in surrounding soils. Because the *Frankia* composition in hosts was non-randomly shaped from sources of free-living *Frankia*, filtering force drove to shape the *Frankia* composition interacting with host alders in a nature. Furthermore, I provided symbiosis filtering index using community

analysis to elucidate the variation in the filtering force among host individuals. The symbiosis filtering indices were more similar among genetically closed alders than other factors, such as soil conditions, geographic distance and community dissimilarity of *Frankia* in rhizosphere soils. This result suggests genetic variation in filtering force of a host plant.

In Chapter 5, to test whether the genetic composition of mutualistic bacteria in host individuals affected growth, survival and preference of an herbivorous insect, feeding and preference experiments were conducted using an alder and a leaf beetle in a field. As a result, I found that *Frankia* composition influenced survival rate of the larval beetle. Moreover, both *Frankia* strains that positively and negatively affect survival of the larvae were detected from the natural *Frankia* composition. These results suggested that an alder interacted with multiple *Frankia* strains which may have different functions on host defense traits.

Integration mutualistic interaction into community genetics

Genomic variation in a foundation species strongly influenced to shape arthropod communities even in a natural forest (Chapter 2). Also, my experiment suggested that genetic variation in an alder (i.e., subpopulations) might affect preference behavior of adults of specialist herbivore insect (Chapter 5). On the other hand, composition of mutualistic bacteria in a host tree affected survival rate of larvae of an herbivorous insect (Chapter 5). These findings suggested possibility of different influences of genetic variation in a host alder and genetic composition of *Frankia* on performance and behavior of a leaf beetle. This suggestion was partially supported by the result that community dissimilarity of *Frankia* was not correlated with genetic distance of an alder (Chapter 4).

Thus, these results suggested that genetic effects of hosts and below-ground symbiont communities can independently influence different aspects in community assembly process of tree-associated herbivorous insects through different phenotype sets. Some trait sets, which are affected by host genetic variation, are likely to affect preferential behavior, leading to influence immigration process in community assembly. Other trait sets, which are affected by symbiont communities, are likely to affect survival, leading to influence filtering process.

One of the candidate trait sets based on host genetic variation would be sensory information traits of host plants to influence immigration process of arthropod communities, such as plant volatile organic compounds (VOCs). For examples, several studies have shown that concentrations and compositions of plant VOCs vary among plant genotypes (Gouinguené et al., 2001; Loughrin et al., 1995; Wason & Hunter, 2014). In contrast, Ballhorn et al. (2013) reported that rhizobia inoculation did not affect concentration of VOCs in legume plants. It is well-known that plant VOCs influence the attraction and behavioral decisions of herbivorous and carnivorous insects (Bolter et al., 1997; De Moraes et al., 1998; 2001; Dicke & van Loon, 2000; Heil, 2004; Horiuchi et al., 2003; Kalberer et al., 2001; Kessler & Baldwin, 2001). I also showed that carnivore communities were likely to depend on genetic identities of a foundation tree (Chapter 2), which may support this pathway that genetic variation in VOC emission influence immigration process in community assembly.

On the other hand, the variation in nitrogen-based defensive traits can be affected by mutualism with nitrogen-fixing bacteria (Ballhorn et al., 2013; 2017; Bauer et al., 2012; Dean et al., 2009; 2014; Katayama et al., 2011; 2014; Kempel et al., 2009; Thamer et al., 2011; Whitaker et al., 2014; Wilson & Stinner, 1984). Host plants store nitrogen

resources from rhizobia as inorganic forms, such as ureides and amides (Berry et al., 2011; Dean et al., 2009; Schubert, 1986). The nitrogen from rhizobia is more useful to produce plant defense than fertilized nitrogen (Dean et al., 2009; 2014). Such direct defense trait sets can be one of critical factors as environmental filters in community assembly process.

Overall, the variation in different plant trait sets discussed above can provide a mechanistic basis for separate effects of plant genetics and symbiont composition on different assembly processes of associated insects. Thus, if genetic variation in a host plant and genetic composition of symbionts independently influence on assembly processes of associated communities, integrating plant genetics and symbiont composition could complementarily develop an understanding of how community forms in complex ecosystems.

Extension of genetic similarity rule by plant–microbe symbioses

Genetic similarity rule, which is advocated as genetically based community assembly rule, is considered to be useful to predict community structure on a plant species, compared to traditional ecological associated assembly rules, such as general ecological rules (Knapp et al., 2004), because genetic identities of a species are produced in long-term selection and evolution, and are likely to be stable during a few years (Bangert et al., 2006). I illustrated that genetic similarity rule is applicable to above-ground arthropod communities in the field, but not to below-ground symbiont communities. Preference and performance of each of above ground arthropod species can be affected by a wide range of traits of host plants, including morphology, growth, nutritional quality, physical traits, and numerous defensive compounds, in the species-specific ways. This complex diversification could be resulted from escape and radiation coevolutionary processes.

Hence, genetic variation associated with these diverse traits can spread throughout host plant genome. Thus, genome-wide SNP variation would also predict community structure well. However, the symbiotic association with members of below-ground bacteria can be strictly determined through stabilization mechanisms and regulated by a few numbers of genes. Different coevolutionary dynamics from escape and radiation could result in the contrasting relationship between genetic variation in hosts and associated communities. The study in Chapter 4 suggested that there is genetic variation in symbiosis filtering of actinorhizal symbiosis even though no significant associations between host genetics and symbiont communities. And, for a decade, previous studies have begun to discover genetic basis underlying actinorhizal symbiosis, including SymRK (Gherbi et al., 2008), CCaMK (Svistoonoff et al., 2013), and NIN gene (Clavijo et al., 2015). These plant genes are known as genetic basis underlying legumes–rhizobia and arbuscular mycorrhizal symbioses and required for nodulation of legumes–rhizobia associations (Froussart et al., 2016). Variation in these mutualism genes may determine the strength and direction of filtering, resulting in shaping symbiont community composition. Consequently, genome-wide SNP variation are not associated with symbiont community structure, but a few functional genes may regulate the community.

This thesis suggests that the integrating mutualistic interactions into the field of community genetics studies will develop genetic similarity rule. For example, the GDM approach in Chapter 2 showed that models with all predictors, i.e., genetic distance of the alder and other factors, remained unexplainable parts of 36.61–63.48 % of deviance in the arthropod community dissimilarity. A focus on mutualistic interactions between a foundation species and microsymbionts may partially explain the residual parts of deviance in the community dissimilarity. Although comprehensive sampling of

rhizosphere symbionts is technically difficult, the above genetic basis in mutualistic interactions of a host plant may be a clue to overcome the difficulty. A merger of the genetic bases of mutualistic interactions with community genetics studies may allow us to understand how communities are formed in complex ecosystems.

Future direction

This thesis suggests that integrating plant–microbe symbiosis into the field of community genetics and ecosystems will improve our understanding for community organization in complex ecosystems. To integrate plant–microbe symbiosis into the community and ecosystems studies, future studies should address the following questions: (1) distinguishing both effects of microsymbiont composition and genetic variation in a host plant on phenotypic variation in the host, and (2) identifying genetic basis of the host associated with shaping above- and below-ground communities. Revealing above questions will lead to understand how community structure shapes in complex ecosystems.

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