Host Specificity of Endophytic Fungi from Stem Tissue of Nature Farming Tomato (Solanum lycopersicum Mill.) in Japan

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Abstract: To understand the distribution of the cultivable fungal community in plant tissues from nature farming tomato plants, we sampled plants of seven different tomato cultivars and recovered 1742 fungal isolates from 1895 stem tissues sampled from three sites in Japan. Overall, the isolation frequency was low (3–13%) and the isolation and colonization frequencies did not vary significantly as a function of the cultivar. The fungi were divided into 29 unique operational taxonomic units (OTUs) with 97% ITS gene sequence identity, the majority of which belong to Ascomycota (99.3%). The dominant genera of cultivable endophytic fungi were Fusarium (45.1%), Alternaria (12.8%), Gibberella (12.0%), and Dipodascus (6.8%). The alpha diversity of the fungal endophytes varied among tomato cultivars. Ordination analysis performed to investigate patterns of endophyte community assemblages on the various cultivars revealed that host cultivars had a significant impact on the endophyte community assemblages in all the study sites. Some of the taxa Fusarium, Alternaria, and Penicillium were found on all cultivars, while few were uniquely present in different cultivars. The dominant taxa may be adapted to the particular microecological and physiological conditions present in tomato stems.

Keywords: colonization frequency; diversity; fungal endophyte; nature farming

1. Introduction

It is now well known that endophytes, organisms that live asymptomatically within plant tissue, are very common and widely distributed in terrestrial ecosystems [1]. The plant and endophytes coevolved millions of years ago [1]. The composition of endophytic fungi is often diversified [2–4], and may vary as a function of host plant species [5,6], geographically distanced individuals of the same host species, and within the tissues or organs of a host plant [7]. In plant-endophyte mutualistic symbiosis, plants provide accommodation and diverse niches for endophytes [8]. In return, many endophytic fungi can improve the uptake and utilization of nutrients by the host to promote growth [9,10] and can enhance tolerance to biotic and abiotic stresses [11–15]. Hence, plant-endophytic
symbiotic associations can play a significant role in the integrity and sustainability of agricultural ecosystems [16]. Therefore, an understanding of the nature, composition and distributions of endophytes is essential both for disclosing features of plant ecology and evolution in ecosystems and for developing further survey schemes for tapping into their functional potentials [17,18].

Conventional farming heavily relies on intensive applications of synthetic agrochemicals and chemical fertilizers, which have negative impacts on terrestrial ecosystems and human health [19]. The cultivation practice in nature farming systems influences plant-associated microbiome diversity and abundance and thus benefits plant growth, development, and health [20–23]. Therefore, to meet the rising global demands of increased eco-friendly farm production, there is a need to develop an agricultural practice that is sound and sustainable for the betterment of both the environment and humans living in it. The ‘Nature farming’, originated in Japan, is a concept that entails a farming practice that does not use synthetic chemical inputs in cultivating crops [24,25]. It is an ecological farming system based on sound agronomic principles and is the major certified organic farming method in Japan [26]. It differs from other organic farming systems primarily in its philosophical origin [27]. More or less similar or comparable farming approaches are in practice in different countries with different names such as ‘Permaculture’ in Australia [28,29], ‘Fertility Farming’ in USA [30], ‘Rishi Kheti’ and ‘Zero Budget Farming’ in India [31,32], Natural Farming in Japan [33], no-tillage farming, zero tillage farming or conservation agriculture in various countries [34]. Introducing nature farming to enhance the predominance of beneficial and effective microorganisms can help improve and maintain soil biological, chemical, and physical properties [35]. This farming concept encourages soil and water conservation, reduces chemical pollution, increases the abundance and diversity of plant and soil microbes, and improves the quality and safety of food supplies [24,26,35,36].

There are no systematic studies of the effects of nature farming practices on the culturable endophytic fungal communities in crop plants. Little is known about the endophyte communities associated with crop plants grown under nature farming, and no study has yet been done on the fungal endophytic communities of nature farming tomato plants. A previous study reported that endophytic microbial species abundance and diversity were significantly higher in organically farmed plants than in conventional plants [37]. While organic farming is not exactly the same as nature farming, but underlying principles overlap. In the current study, we aimed to identify culturable fungal isolates that are likely to be beneficial to plant growth, health, and yields and to reveal whether those endophytes vary in different tomato cultivars. This study represents basic information about the occurrence and species diversity of fungal endophytes in the stems of tomato plants growing in nature farming in Japan. The advantage of the culturable approach is that it allows isolation, identification, and consequently may contribute to further studies of function and application of endophytic fungi. [12,38,39]. As such, its findings might aid the development of novel, efficient, and sustainable strategies for improving crop quality and productivity while reducing the application of harmful chemicals.

2. Materials and Methods

2.1. Collection of Tomato Plant Samples

Nature farming tomato plants (Solanum lycopersicum L.) were collected from four different experimental farmlands (Site-C: the nature farming began in 1990, Site-D: the nature farming began in 1992 and Site-I: the nature farming began in 1999, distances between Site-C and Site-D: 1.8 km, between Site-D and Site-I: 0.4 km, between Site-I and Site-C: 1.6 km, respectively) in International Nature Farming Research Center (INFRC), Matsumoto, Nagano, Japan. The soil type in all experimental sites is andosol. In these experimental fields, instead of pesticides and chemical fertilizers, compost and green manure are applied to maintain the soil fertility and the ecological balance of farmlands. The tomato plants comprised several cultivars and were grown under different cultivation practices. Plant are of similar age (late fruiting stage) and three plants from each cultivar per cultivation practice.
per site were sampled in August 2019. After harvesting, the plants were packed in polythene, shipped in an ice box, stored in a refrigerator until use and processing was done within 72 h. Details of the cultivars are presented in Table 1.

Table 1. Natural farming tomato (Solanum lycopersicum) cultivars used for the isolation of stem endophytes. These sites belong to International Nature Farming Center (INFC); 5632-1 Hata, Matsumoto, Nagano, Japan.

| Site  | Cultivation Practice          | Cultivar                                      |
|-------|-------------------------------|------------------------------------------------|
| Site-C| Living Mulch (Grass Cultivation) | Chika, Menina, Momotaro8, Mountain Fresh Plus-M, Myoko, Rosso Neapolitan |
| Site-D| Living Mulch (Grass Cultivation) | Chako, Rosso Neapolitan                       |
| Site-D| Bare Field                    | Chako, Rosso Neapolitan                       |
| Site-I| Green Manuring                | Chako, Chika, Menina                          |

2.2. Fungal Endophyte Isolation

We harvested whole plants and stems were cut from the lower part of the main stem from above 10 cm off the ground. The leaves, secondary branches and approximately 10 cm from the top were removed before endophyte isolation from the main stems to reduce heterogeneity among the samples. Asymptomatic tissues from the main stem of the tomato plants were used for endophyte isolation. Tissues were washed under running tap water to remove surface debris before surface sterilization and cut into ~5 cm long segments for ease in processing. Stem tissues were surface-sterilized by immersion in 0.5% sodium hypochlorite for 3 min before immersion in 70% ethanol for 3 min. After three washes in sterile distilled water and blotting with sterile filter paper, samples were cut into approximately 3 x 3 mm segments with a sterile scalpel. We placed around 50 segments (3 x 3 mm) from each plant and there were three plants for each cultivar per cultivation practice per site with a missing plant of the cultivar ‘Chako’ at site-D (~50 segments x 13 plants x 3 replications minus 1 missing plant), which made a total of 1895 segments (supposed to be 1900 segments but some plants had 1 or 2 missing segments due to mistake). To evaluate the efficacy of the surface sterilization procedure, the imprint method was used, i.e., pressing sterilized tissue segments gently onto potato dextrose agar (PDA) [40] to confirm that the fungal isolates only originated from the internal tissues of the tomato stem segments. Additionally, the water that was used to wash the surface sterilized tissues at the last step was incubated in 0.25 x PDA to observe any fungal growth. For fungal isolation, segments were plated in petri dishes containing media composed of 0.1 x PDA (PDB 2.4 g (Catalog # P6685, Difco) plus 17 g agar in 1 L water amended with 100 µg/mL streptomycin sulfate (Sigma, St Louis, MO, USA). Each plate contained five stem tissue fragments and was incubated at 25 °C in the dark and checked periodically for fungal growth. The growing margins (hyphal tips) of colonies from tissue segments were transferred to plates of 0.25 x PDA supplemented with streptomycin (100 µg/mL) [41]. The fungal isolates were counted and stored temporarily at 4 °C, and the mother culture was permanently stored at −80 °C in 80% sterile glycerol.

2.3. Endophyte Identification

We employed a combination of morphological and molecular techniques for the identification of fungal taxonomy. Fungi that showed similar morphological characteristics, such as colony characteristics and mycelial textures, in our culture plates were grouped, and one of them was identified as a representative strain. For molecular identification, fungal mycelium taken from an agar plate was transferred into a 50 mL falcon tube containing 20 mL PDB and incubated on a shaker for 7–21 days in the dark. At harvest, the fungal mycelium was trapped by a sterile mesh strainer and squeezed dry on a sterile paper towel and used for genomic DNA isolation by following a phenol:chloroform:isoamyl alcohol (PCI, 24:24:1) protocol [5]. Briefly, cryogenically ground fungal mycelia (approximately 100 mg) were lysed in 450 µL of extraction buffer (0.2 M Tris–HCl pH 7.5,
0.25 M EDTA, 0.2 M NaCl, 1% SDS) and mixed thoroughly with continuous shaking for 5 min. An equal volume of PCI solution was added to the lysate and incubated for 5 min after mixing and centrifuged at 20,000× g for 5 min. Then, 300 µL of the supernatant was transferred to a 1.5 mL tube. The genomic DNA was precipitated with an equal volume of iso-propanol at −20 °C for 15–30 min followed by centrifugation at 14,000× g for 20 min at 4 °C. The pellet was washed with 70% ethanol, air dried and dissolved in 50 µL of EB buffer (10 mM Tris-Cl, pH 8.5).

We amplified the ITS regions of the fungi using the universal primers ITS1 and ITS4 [42], using the PCR conditions as 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min. The 20 µL reaction mixture contained 10 µL of 2 × Promega Go Taq™ Master Mix, 10 µM of each primer, 1 µL of template DNA and 7 µL of water. Amplicons were purified by an AxyPrep Mag PCR Clean-up kit (Axygen Biosciences, Union City, CA, USA). Each sequencing reaction contained 1 µL of BigDye® Terminator sequence mix (Applied Biosystems, Foster City, CA, USA), 1.5 µL of 5 × Sequencing Buffer, 1 µL of the forward primer (3.2 µM), 4 µL cleaned PCR amplicons and 2.5 µL water. The cycling conditions were 35 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min.

The fungal ITS sequences were viewed for quality checking by using FinchTV 1.5 (Geospiza Inc., Seattle, WA; MacOS X), first ~20nt were trimmed, any ambiguous bases (N) were manually corrected by matching the chromatogram and average 500–800 bp high-quality sequences were aligned. The sequences were aligned as query sequences with the database UNITE using massBLASTER analysis options, which is specially compiled and used for fungal identification [43,44]. In all cases, the isolates were identified to the species level if their sequences were >98% similar to any identified accession from the database. When the similarity percentage was between 95–97%, only the genus name was accepted; for sequence identities <95%, the isolates were classified according to family or order or as “unknown fungus” [5]. The isolates that matched the same taxa were aligned using the MAFFT (multiple alignment using fast Fourier transform) algorithm [45], and percent similarity was obtained using the EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/mafft/) platform. A ≥97% similarity threshold was used to distinguish taxa that yielded the same blast hit and noted them as “A” or “B” at the end of taxa name. The sequences were deposited in GenBank, and accession numbers were obtained (MT481755-MT481800).

Phylogenetic analysis of the isolated endophyte genera obtained from nature farming tomato plants. The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura–Nei model [46]. The tree with the highest log likelihood (−8023.30) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with a superior log likelihood value. This analysis involved 46 nucleotide sequences. The codon positions included were 1st+2nd+3rd+noncoding. There were a total of 1003 positions in the final dataset. Evolutionary analyses were conducted in MEGA X 10.1.5 [47].

2.4. Colonization and Isolation Frequency

Colonization frequency (CF) was defined as the percentage of tissue fragments yielding an endophyte in culture; on the other hand, the isolation frequency (IF) was defined as the number of taxa isolated from a cultivar in a particular site and expressed as the percentage (%) of tissue planted. At first, we checked all data if these met the assumptions of normal distribution and homogeneity of variance using the Shapiro–Wilk normality test and Levene’s test, respectively. Colonization frequency (CF) data form Site-C and Site-I were found to be violating the assumption of normality and we used the Kruskal–Wallis (non-parametric) test for comparisons (Table S1). In all other cases, ANOVA tests were carried out to compare the CF and IF values between cultivation practices at site-D, which revealed no differences between them and their interaction with cultivars. Next, we conducted ANOVA tests
among host genotypes at different sites and Tukey’s HSD test for multiple comparisons. We used the following formulae for calculating CF and IF:

\[
CF(\%) = \frac{\text{Number of segments shows endophyte growth}}{\text{number of segments plated}} \times 100
\]

\[
IF \text{ (IF\%)} = \frac{\text{Number of unique taxa isolated}}{\text{number of segments plated}} \times 100
\]

2.5. Diversity and Community Composition

The diversity measures used were the Shannon–Wiener diversity index (H’), Fisher’s alpha diversity, evenness (H/S), Menhinick index and Simpson dominance. These were used to measure endophyte species diversity within samples (α-diversity). Since the results were consistent in all cases, only the Shannon–Wiener biodiversity, the most commonly used index, has been shown in the findings index (H’). An ANOVA test was performed to compare H’ values among host genotypes and cultivation practices.

We compared differences in community composition and structure among host cultivars (β-diversity) using analysis of similarity (ANOSIM) and PERMANOVA. The use of two different pairwise similarity measures was attempted because they take into account different types of information that can provide different insights for comparing communities [48]. We calculated (i) Jaccard’s index, which considers only the presence or absence of fungal taxa among samples, and (ii) the Bray–Curtis coefficient, which considers the abundance of taxa along with the presence or absence of particular fungal taxa [49].

Nonmetric multidimensional scale (NMDS) ordination was applied to visualize similarity measures of endophyte communities. Multidimensional scaling is used to graphically present interactions between objects in multidimensional space. NMDS is a popular and robust visual analysis tool applicable to various data types, and it is useful to a number of user-defined standardizations and transformations of the data, flexible with respect to which dissimilarity or similarity measure is used [48]. NMDS plots use rank-order information in a dissimilarity matrix [50]. The test statistic “R” and “pseudo-F” values were calculated as the difference of mean ranks between vs. within groups. Significance was calculated by 10,000 permutations of group membership. The diversity measures (α-diversity and β-diversity including NMDS plots) analyses were conducted using PAST ver. 3.26 [51].

3. Results

3.1. Isolation and Identification of Endophytes

The efficacy surface sterilization was confirmed by the absence of fungal or bacterial growth in tissue imprint, which indicated that the surface sterilization protocol was effective in eliminating epiphytic fungi. A total of 1742 isolates of fungal endophytes were recovered from 1895 stem segments of different cultivars of tomato plants from three sampling experimental sites (overall colonization frequency = 91.926%). Here, an isolate is defined as visible fungal growth from a (3 × 3 mm²) stem segment/plant/cultivar/location. The ANOVA test of colonization frequencies indicated that the number of fungal isolates recovered did not vary significantly as a function of cultivar except at site D, where ‘Chako’ yielded a significantly lower number of isolates than ‘Rosso Neapolitan’ (p = 0.003, Figure 1). Isolation frequency (IF), which we defined as the mean number of endophytic taxa per number of segments plated per cultivar in percentage, did not vary among cultivars at Site-D and Site-I (Figure 2). At Site-C, the Mountain Fresh Plus-M cultivar had the lowest IF, which was significantly different from that of Rosso Neapolitan. Overall, the isolation frequency was low (3–13%) in all cultivars regardless of the collection site (Figure 2). We did not observe any difference in endophyte CF or IF due to different cultivation practices at site D (Tables S2 and S3).
Figure 1. Colonization frequency (CF) of fungal endophyte of the stem of nature farming tomato (*Solanum lycopersicum*) at three experimental sites in the INFRC. The CF refers to the mean number of tissues colonized in a cultivar and expressed as percentage (%) of tissue planted in PDA plate. The error bar indicates the standard error (SE) of the mean. The *p*-values were obtained from Kruskal–Wallis (Site-C and I) or ANOVA (Site-D) to indicate significant differences.

Figure 2. Isolation frequency (IF) of fungal endophytes from the stem of nature farming tomato (*Solanum lycopersicum*) at three different locations in Japan. The IF refers to the mean number of taxa isolated from a cultivar and expressed as percentage (%) of tissue plated in PDA plates. The error bar indicates the standard error (SE) of the mean. The *p*-values were obtained from ANOVA (Site-D and Site-I) and by post hoc test using Tukey’s HSD test (Site-C) to indicate significant differences (different letters above the bars).

In aggregates across all sampling locations, 46 representative fungal isolates were selected for molecular identification based on morphological characteristics in cultures. Molecular identification using ITS rDNA sequences resulted in a total of 29 different endophytic fungal taxa that belonged to 12 different genera with few taxa not identified to the genus level. All endophytes recovered from tomato cultivars were members of Dikarya. Ninety-nine percent of endophyte isolates recovered here represented Ascomycota and were distributed in four classes of Pezizomycotina. The majority represented Hypocreales (59%), Pleosporales (21%), and Saccharomycetales (7%) (Figure 3). Overall, the most dominant fungal genera were *Fusarium*, *Gibberella*, *Dipodacus*, and *Alternaria* (Figure 3). Phylogenetically, the fungal endophytes recovered from our nature farming tomato plants did not seem highly diverse (Figure 4).
3.2. Diversity and Species Richness of Endophyte Communities (α-Diversity)

The diversity of each endophytic fungal community was assessed in relation to host cultivar and cultivation practice using the Shannon–Wiener diversity index (H') value. We also did not detect any difference in alpha-diversity of fungal endophytes among the cultivars at Site-D and Site-I, but there was a variation at site-C. There was significantly higher fungal diversity recorded from the cultivars Menina and Chika (H' ≥ 1.0) than from the cultivars Momotaro8, Mountain Fresh Plus-M, and Rosso Neapolitan (H' ≤ 0.3) at site C (Figure 5). The diversity of the endophyte communities did not differ significantly between the plot with previously grass cultivated and bare plot without grass cultivation (Figure 5).

3.3. Variation in Endophyte Communities across Location, Host and Tissue (β-Diversity)

Ordination analysis was performed to investigate patterns of endophyte community assemblages on the various host cultivars. Two-dimensional nonmetric multidimensional scale (NMDS) as well as ANOSIM- and PERMANOVA-based clustering of fungal communities revealed that cultivation practice (that is, living mulch intercropping vs. bare field) did not influence the structures of endophyte communities at sampling site D (R = 0.069, p = 0.2603; Figure 6). However, host cultivars had a significant impact on the endophyte community assemblages in all study sites (Figure 7). The ANOSIM statistic R ranged from 0.45 to 0.95, indicating a dissimilarity of the endophyte communities among the host cultivars and that the endophyte communities at particular locations appeared to be specific to particular host cultivars (Figure 7).
Figure 4. Phylogenetic analysis of the isolated endophyte genera obtained from nature farming tomato plants. The evolutionary history was inferred using the Maximum Likelihood method and Tamura–Nei model [46]. The tree with the highest log likelihood (−8023.30) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 46 nucleotide sequences. The codon positions included were 1st+2nd+3rd+noncoding. There were a total of 1003 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [47]. The “A” or “B” at the end of the taxa name are given to distinguish taxa of the same name with an ITS sequence dissimilarity of >3%.
Figure 5. Fungal endophyte biodiversity analysis. The effects of host cultivar and cultivation practice on fungal endophyte biodiversity as measured by the Shannon–Wiener index (H’). Endophytes were isolated from the stems of different tomato cultivars grown under nature farming conditions and sampled from three experimental sites in INFRC. The Within each site, different letters (a,b) above the bars indicate significant differences (p < 0.05, ANOVA and Tukey’s HSD tests).

Figure 6. Fungal endophyte communities as influenced by cultivation practice. Nonmetric multidimensional scaling (NMDS) and ANOSIM analysis of endophyte communities recovered from site-D from two cultivation practices as indicated by different colors as well as labels. For clustering analysis, two different community similarity indices were computed: (a) Bray–Curtis coefficient, which compares fungal taxa presence or absence along with the abundance among groups, and (b) Jaccard’s index uses only presence and absence data for comparing fungal community similarity among groups. The ANOSIM statistic R and The PERMANOVA statistic F values and the corresponding p-values indicating the significance of dissimilarity were obtained by permutation of group membership, with 9999 replicates.
Figure 7. Fungal endophyte communities as influenced by host cultivars. Nonmetric multidimensional scaling (NMDS), one-way ANOSIM and one-way PERMANOVA analyses of endophyte communities recovered from several tomato cultivars as indicated by colors and labels at different locations in Japan. For clustering analysis, two different community similarity indices were computed: (a,c,e) Bray–Curtis coefficient, which compares fungal taxa presence or absence along with the abundance among groups, and (b,d,f) Jaccard’s index uses only presence and absence data for comparing fungal community similarity among groups. The ANOSIM statistic $R$ values; large positive $R$ (up to 1) signifies dissimilarity between groups. $F$ indicates the pseudo-$F$ values. *** and ** represent the significance of dissimilarity ($R$) at $p < 0.001$ and $p < 0.01$, respectively, obtained by permutation of group membership, with 9999 replicates. RN = Rosso Neapolitan, MF = Mountain Fresh Plus-M.
4. Discussion

Microbial symbionts of plants, including endophytes, have increasingly received attention in agriculture and biotechnology [4,53–58]. Fungal endophyte communities have been explored from a broad range of wild and agricultural plants. The object of our current study was to examine for the first time the composition, biodiversity and host affiliation of tomato plants grown under nature farming.

Most published studies have focused on root or leaf endophytes and those from wild or conventionally cultivated crops, but limited numbers of studies have reported on crops grown with nature farming. The present study targeted stem fungal endophytes obtained from different tomato cultivar plants cultivated in nature farming. The resulting data set will contribute to the augmentation of the available knowledge on shoot-associated fungal endophytes of plants, especially those of nature farming.

The techniques used for endophyte isolation are important considerations [57]. The accuracy of identification and isolation is dependent on various factors that, in turn, may affect the usability and validity of datasets for comparison among groups. Too harsh surface sterilization procedures might kill all endophytes in plant tissues [58], whereas improper sterilization will allow epiphytes to proliferate and contaminate and thus reduce the ability to recover viable isolates grown on culture media. We used tissue imprinting to rule out the possibility of contaminants and optimize the surface sterilization procedure [59]. Again, we use only PDA media, which might also have an impact on the isolation of endophytes because some isolates might not grow on that media. However, since the same sterilization protocol, media and growth conditions were applied to all samples in this study, we expect that any bias related to the above-described was masked and gave us good confidence for comparable analyses. There are also problems in the identification of culturable endophytes using ITS amplification of DNA barcoding approaches [60,61]. Certain taxa can only be identified to the family or higher levels due to the absence of resembled sequences in GenBank [62]. In addition, the sequences deposited in the GenBank database, we used the UNITE database, which is specifically curated as described by other studies [44,63,64]. There are also concerns that ‘endophyte’ could be ‘latent pathogen’. Indeed, fungi isolated as endophytes have been shown to produce disease in the host upon inoculation [65–68]. In this paper, we did not evaluate the pathogenicity of any of the isolates obtained but we relied on the well-accepted definition of endophytes. To be specific, we used the term ‘endophyte’ to refer to the fungi that were present within the healthy plant tissue, rather than on the surface, without showing apparent disease symptoms or without necessarily implying any specific ecological functions [67–69].

4.1. Composition of Endophytes

On average, endophytes were isolated in culture from 92% of the tomato stem tissue segments plated. This seems to be very high colonization rather than those reported from other studies. This may be an implication of a possible higher colonization intensity of fungal endophytes in nature farming crops than conventional cropping or wild plants. Indeed, previous studies that compared fungal endophytes of tomato, soybean and grapevines grown organically reported higher overall colonization than those grown under conventional practice [12,70–72]. However, further studies to compare endophyte isolation from the same crop grown under different cultivation systems may reveal the potential differences and the cause of differences in colonization intensity.

DNA sequence data revealed 29 distinct fungal OTUs, of which the majority belonged to the phylum Ascomycota (92%). The predominance of Ascomycetes is likely to be characteristic of endophytic mycota identified from other plant species. Phylum Basidiomycetes have also been reported as endophytes in many plant species, although, as we found, in lower numbers and frequencies in this study [5,60]. Phylogenetically, the fungal endophytes recovered from our nature farming tomato plants seemed not highly diverse (Figure 7). Fusarium was the most dominant genus in all tomato cultivars, and most of them were very closely related and are grouped into three common evolutionary
groups. Another dominant genus was *Alternaria*, and several species were very closely related and had a common origin. The dominant endophytic fungi identified in this study contained several isolates of the genera *Fusarium*, *Alternaria*, *Aspergillus*, and *Penicillium*; most of them represent common saprophytes or plant pathogens. However, they were also reported to be common plant symbionts playing significant roles in plant growth promotion, such as nutrient acquisition, tolerance to harsh environments, and defense against pathogens and herbivores [14,73–77].

Our results are in accordance with previous studies [21,75], which reported the frequent occurrence of species of *Alternaria* sp., *Aspergillus*, *Chaetomium*, *Fusarium*, and *Penicillium* sp., isolated from tissues of tomato plants. Xia et al. [21] also identified certain other endophytic fungi, such as *Nigrospora*, *Periconia*, and *Hypoxylon*, from both organic and conventionally grown tomato in the USA that were not detected in our study. This indicates that internationally, *Nicotiana* species may be colonized by a wider range of endophytic fungal taxa.

4.2. Diversity and Structuring of the Endophyte Community

Endophytic composition and diversity can be shaped by several factors, such as host genotypes, tissue origin, local environmental conditions, nutrient availability and interactions with soil fungi and bacteria [5,76–81]. Therefore, we predicted that endophytic composition and diversity would exhibit substantial variation among different tomato cultivars. The Shannon–Wiener diversity index ($H'$) values, which consider both richness (the number of species) and evenness (relative abundance) of the individuals present in a sample [82], were not significantly different among cultivars in two locations, but we did observe a difference at one location (Site-C), implying that host genotype may or may not impact the diversity of endophytes relying on geography, farming practice or other factors.

The isolation frequency as well as the diversity of the endophytes was relatively low. Previous studies also indicated that culturable, horizontally transmitted endophytes in aboveground plant parts are often negatively correlated with factors such as UV radiation and aridity [59,83] and that endophytes colonizing stem and leaf tissues of these plants are not as diverse as those inhibiting belowground tissue [5,61,84–86]. We do not extend our hypothesis that root tissues might have higher endophytic diversity since we did not study endophyte colonization in root tissues, but further study may reveal this fact.

Within a particular sampling location, we attempted to examine the effect of host cultivars on endophyte community structures by comparing three different indices of community similarity ($\beta$-diversity), each of which conveys different information and explanations. The Bray–Curtis coefficient uses quantitative species abundance data, while Jaccard’s similarity measure takes into account only binary presence–absence data [49,87]. Irrespective of the community similarity index employed, we observed a clear clustering of endophyte communities associated with different tomato genotypes at any of the sample locations (Figure 7). This implied that the endophyte community structure can be highly influenced by host genotypes. Regarding cultivation practices, we did not observe any clustering due to different cultivation practices, indicating neutral effects of cultivation practices on endophyte community structures at any particular location.

This is the first report of endophytic fungi from nature farming tomato from multiple sites. The results obtained in this work are in agreement with reports on fungal endophytes in which higher colonization is described with few species observed frequently (dominant). Some of the taxa *Fusarium*, *Alternaria*, and *Penicillium* were found on all cultivars, while few were uniquely present in different cultivars. The dominant taxa may be adapted to the particular microecological and physiological conditions present in tomato stems. However, it should be noted that our inferences have limitations because we focused only on cultivable fungi, and specifically, on those that could be cultured on PDA, we are likely to have underestimated the numbers of fungi present in our samples. Previous research indicates that plants harbor many species of endophytes, including other microbiomes that remain undetected in culture-based approaches but can be detected in culture-independent approaches [88–90]. Such tools may be particularly important for crops growing in natural or organic systems since less
human intervention in cropping is expected to increase the diversity and community composition of plant-associated microbes. Therefore, culture-independent identification approaches should ideally be combined with culture-dependent methods. Future work could also include a study of the association (if any) of these fungi, particularly the dominant fungal genera such as *Fusarium*, *Alternaria*, *Penicillium* and *Aspergillus*, on the growth and development of their host. Nevertheless, information on endophytic fungal assemblages in plants could serve as a database reference for assessing fungal diversity from other geographical locations. Various diversity indices exhibited the diversity of the fungal endophytic population in relation to the abovementioned factors. Our study will help in understanding the ecology and community structure of endophytic fungal associations in nature farming crops. We did not investigate the sexual reproduction of any of the isolates obtained in the current study but future study might consider examining the production of sexual spore along with other mycological investigations for better understanding the endophyte, their characteristics and roles in a host.

The roles of fungal endophytes are diverse and vary widely. They have been described as assisting plants in the uptake of nutrition and protecting against herbivory, plant pathogens and environmental stress [54,55]. Furthermore, nature is a great source of diverse chemical compounds with huge bioactive prospects that cannot be recreated in laboratories. Endophytic fungi are relatively less explored natural resources that can be a promising source of secondary metabolites. Future experiments might undertake works focusing on identifying the potential roles of endophyte candidates and detecting the metabolites, as a first step towards evaluating their eco-physiological importance in sustainable agriculture.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/7/1019/s1, Table S1. Results of tests for normality of distribution and homogeneity statistics, Table S2: Two-way ANOVA test showing the effect of cultivation practice and cultivar on fungal endophyte colonization frequency from the stem of nature farming tomato (*Solanum lycopersicum*) at Site-D in Japan, Table S3: Two-way ANOVA test showing the effect of cultivation practice and cultivar on fungal endophyte isolation frequency from the stem of nature farming tomato (*Solanum lycopersicum*) at Site-D in Japan.

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