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Research Article

Cutaneous Microflora from Geographically Isolated Groups of *Bradysia agrestis,* an Insect Vector of Diverse Plant Pathogens

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Abstract Larvae of *Bradysia agrestis,* an insect vector that transports plant pathogens, were sampled from geographically isolated regions in Korea to identify their cutaneous fungal and bacterial flora. Sampled areas were chosen within the distribution range of *B. agrestis*; each site was more than 91 km apart to ensure geographical segregation. We isolated 76 microbial (fungi and bacteria) strains (site 1, 29; site 2, 29; site 3, 18 strains) that were identified on the basis of morphological differences. Species identification was molecularly confirmed by determination of universal fungal internal transcribed spacer and bacterial 16S rRNA gene sequences in comparison to sequences in the EzTaxon database and the NCBI GenBank database, and their phylogenetic relationships were determined. The fungal isolates belonged to 2 phyla, 5 classes, and 7 genera; bacterial species belonged to 23 genera and 32 species. Microbial diversity differed significantly among the geographical groups with respect to Margalef's richness (3.9, 3.6, and 4.5), Menhinick's index (2.65, 2.46, and 3.30), Simpson's index (0.06, 0.12, and 0.01), and Shannon's index (2.50, 2.17, and 2.58). Although the microbial genera distribution or diversity values clearly varied among geographical groups, common genera were identified in all groups, including the fungal genus *Cladosporium*, and the bacterial genera *Bacillus* and *Rhodococcus*. According to classic principles of co-evolutionary relationship, these genera might have a closer association with their host insect vector *B. agrestis* than other genera identified. Some cutaneous bacterial genera (e.g., *Pseudomonas*) displaying weak interdependency with insect vectors may be hazardous to agricultural environments via mechanical transmission via *B. agrestis*. This study provides comprehensive information regarding the cutaneous microflora of *B. agrestis*, which can help in the control of such pests for crop management.

Keywords Biodiversity, *Bradysia agrestis*, Insect microflora, Insect vector

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Global warming has inevitably led to problems in agricultural environments, including an increase in crop diseases transmitted by insect vectors, resulting in severe economic problems akin to the challenges of human vector-borne diseases [1-7]. Environmentally friendly agricultural practices have increased over time owing to rising consumer awareness of national and global food security requirements. However, given the concentration of agricultural environment in East Asia, it is difficult to find solutions for pest control that do not involve agricultural pesticides. Thus, implementation of environmentally friendly agricultural practices has become extremely challenging because of the emerging insect vector crisis.

The insect vector *Bradysia agrestis* represents an important emerging agricultural concern in East Asia given that it is responsible for the spread of significant infective fungal

genera such as *Fusarium*, *Phoma*, *Pythium*, and *Verticillium* among commercial crops [2]. *B. agrestis* causes the most substantial damage during the larval stage. Larvae of the insect vector flourish in the rhizosphere, and their caterpillars feed on organic matter, decayed plant debris, and the colonies of fungi such as oomycetes, ascomycetes, and basidiomycetes [8, 9]. Larvae of this species can uniquely transmit several fungal diseases such as *Fusarium* blight via the release of fungal colonies from the inner tissues of the plant [8, 10]. In other words, they are capable of inducing both mechanical damage and secondary infection [10]. However, the entire cutaneous microflora of this vector, which could be mechanically transferred by disrupting the plant surface barrier, has not been well studied.

To date, most studies have only identified the transfer of classically well-known fungal strains. This strategy represents a type of post-facto management, which occurs following serious outbreaks. However, revealing the entire microbiota that could be transferred by the host vector would be a pro-active management strategy to minimize agricultural emergencies. Furthermore, the entire microflora is closely associated with and greatly affects the physiology, life cycle, reproduction, and immunity of host vectors. Therefore, this information is essential for combatting emerging vectors. In particular, comparing the microbial flora of geographically segregated vector populations may provide important information for controlling insect vectors and preventing possible fungal disease outbreaks [3, 4, 6, 11-14].

Our research team has performed comprehensive investigations of the gut fungal flora that induces biological transmission in *B. agrestis*, with special focus of Korean populations [10, 15]. In the present study, we aimed to identify the major cutaneous fungal and bacterial flora associated with mechanical transmission or secondary infection via mechanical damage, as part of the national public project to address the severe *B. agrestis* blight in Korea. The community structures, geological distribution, and microbial diversity of cutaneous fungal and bacterial flora of *B. agrestis* were investigated. Furthermore, the interdependency between the associated species and their host vectors, as it relates to the potential impact on agriculture, was assessed.

MATERIALS AND METHODS

Collection and treatment. Three regional groups (sites 1 to 3) (Table 1, Fig. 1) were sampled from September 16 to November 5 of 2016, which corresponds to the outbreak season for *B. agrestis* [10]. Each geographical group was geographically segregated (greater than 91 km apart in distance), and the possibility for ecological interaction was minimal [16]. A standard dipping technique was applied for larval sample collection using a 350-mL dipper [7]. The dippers were sterilized using ethanol (EtOH), and ultraviolet (UV) light was used to control microbial contamination [7]. Sixty larval samples were collected from each region, and *B. agrestis* specimens were identified by morpho-taxonomic methods [17]. Larvae samples (180 from each site) were transferred to the laboratory and immobilized by incubating

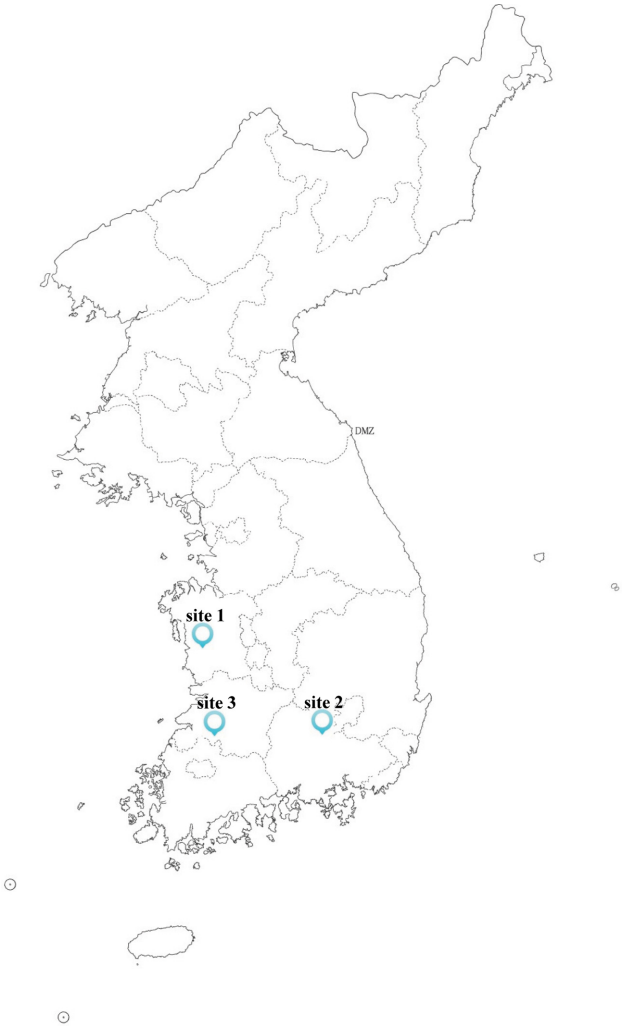


Fig. 1. Location of sampled sites in each geographical vector group in Korea. Site 1, Sedo-myeon, Buyeo-gun, and Chungcheongnam-do, Republic of Korea; Site 2, Ssangchaek-myeon, Hapcheon-gun, Gyeongsangnam-do; Site 3, Iseo-myeon, Wanju-gun, Jeollabuk-do.

Table 1. Sampling information for three geographically distant regions

	Geographical group	Vegetation	Isolate counting
Site 1	Sedo-myeon, Buyeo-gun, Chungcheongnam-do, Republic of Korea	Tomato and lily	Fungi (5)/bacteria (24)
Site 2	Ssangchaek-myeon, Hapcheon-gun, Gyeongsangnam-do, Republic of Korea	Strawberry	Fungi (3)/bacteria (26)
Site 3	Iseo-myeon, Wanju-gun, Jeollabuk-do, Republic of Korea	Tomato and lily	Fungi (5)/bacteria (13)

at −20°C for 10 min. For isolation of cutaneous microbes, the samples were first sprayed with a cold sterile insect saline solution (9.32 g NaCl, 0.77 g KCl, 0.5 g $CaCl_2$, 0.18 g $NaHCO_3$, 0.01 g NaH_2PO_4, pH 7.4 in 1 L of distilled water) to eliminate foreign substances. They were then affixed to paraffin-filled dishes covered with insect saline solution. The skin of larvae was isolated from the body using a dissecting microscope. Isolated skin samples were homogenized with a mortar in a 2-mL Eppendorf tube with 150 µL insect saline.

Fungal isolation and identification. For fungal isolation, serially diluted insect saline was spread onto potato dextrose broth (PDB; BD Difco, Franklin Lanes, NJ, USA) agar medium containing 80 mg/L streptomycin (Sigma-Aldrich, St. Louis, MO, USA). All media were incubated at 25°C for 14 days in the dark [18]. Serial subcultures were performed to obtain pure isolates. For partial molecular identification of all fungal isolates from the cutaneous microflora of *B. agrestis*, the internal transcribed spacer (ITS) rDNA sequences were analyzed. The isolates were inoculated onto the PDB (Difco, Detroit, MI, USA) agar plates and incubated at 25°C for 7 days on a rotary shaker at 120 rpm. Filtered mycobionts were lyophilized for 2 days, and then genomic DNA was extracted from the lyophilized mycobionts using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). Primers targeting the ITS regions ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used for amplification [19]. The PCR cycling conditions were as follows: pre-denaturation (94°C, 4 min); denaturation (94°C, 1 min), annealing (55–58°C, 1 min), and extension (72°C, 2 min) for a total of 35 cycles; and final extension (72°C, 2 min) [20]. PCR products were confirmed by electrophoresis (1.5% agarose gel, stained with ethidium bromide [EtBr]), and the resulting bands were visualized using a UV transilluminator. The AccuPrep PCR & Gel Extraction Kit (Bioneer, Daejeon, Korea) was used to purify PCR products, and an ABI 3730XL DNA analyzer (Applied Biosystems, Carlsbad, CA, USA) was used for analysis [18]. To determine the taxonomic position of the isolates, the ITS sequences were compared with similar sequences from other fungal species in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) using the BLASTn tool [20]. The NCBI GenBank accession numbers for the fungal sequences are KY929272–KY929284 (Supplementary Table 1).

Bacterial isolation and identification. For bacterial isolation and identification, the diluted supernatant (10^{-4}–10^{-6}) of the homogenized skin was spread onto 1/10 diluted tryptic soy broth (TSB; Difco) and nutrient broth (NB; Difco) agar plates. Each plate was incubated at 25°C for 72 hr. Thereafter, isolates from the 1/10 TSB and NB agar were streaked onto non-diluted TSB agar plates to exclude morphologically identical isolates. Isolates from the 1/10 TSB or NB agar medium were routinely cultured in non-diluted TSB. For molecular identification of the isolates, the cultures were grown in 50 mL TSB liquid media, with shaking at 150 rpm. Bacterial cells were collected by centrifugation at 10,000 ×g for 10 min. To obtain genomic DNA, a boiling method with Chelex Resin (Bio-Rad, Hercules, CA, USA) was applied, and 16S rRNA gene sequences were amplified using the primer pair 27-F (forward primer 5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492-R (reverse primer 5′-GGT TAC CTT GTT ACG ACT T-3′). For sequential PCR, an initial denaturation step (95°C, 15 min) was performed, followed by denaturation (95°C, 20 sec), annealing (50–58°C, 40 sec), and elongation (72°C, 90 sec) steps (30 cycles), and a final elongation step (72°C, 5 min). Amplified PCR products were precipitated in EtOH (65%) and sodium acetate (3 M, pH 4.6) for 15 min, and centrifuged at 2,000 ×g for 45 min. The supernatants were discarded, and the samples were centrifuged in 70% EtOH (10,000 ×g, 10 min). This process was repeated 7 or 8 times. The PCR products were purified using the Gel and PCR Extraction System-UB, and sequence analysis was performed using an ABI-3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) [21, 22]. The acquired final partial 16S rRNA gene sequences were compared with sequences previously deposited in the Ez-Taxon database (http://eztaxon-e.ezbiocloud.net). BLAST was used to identify sequence similarities between strains [21-23]. The NCBI GenBank accession numbers for the bacterial sequences are KU711995–KU712065 (Supplementary Table 1).

Phylogenetic analysis. To determine the phylogenetic relationship of the isolates, partial fungal ITS sequences or bacterial 16S rRNA genes were aligned using the Clustal X program [24], and arrayed by the BioEdit sequence alignment editor [25]. Phylogenetic trees were constructed based on the Kimura 2-parameter model via the MEGA 6.0 maximum likelihood algorithm [26, 27]. The stability of relationships

Table 2. Diversity index formulae used in this study

Diversity indices	Formula	Description
Shannon's diversity index (H′)	$H' = \sum_{I=1}^{R} pi \cdot \ln pi$	ni, the number of clones in the ith OUT
Simpson's index of diversity (1-D)	$D = \sum_{I=1}^{R} ni(ni-1)/N(N-1)$	N, total number of the individuals in each sample
Menhinick's index (Dmn)	$Dmn = S/\sqrt{N}$	pi, ni over N
Margalef's index (Dmg)	$Dmg = (S-1)/\ln(N)$	S, the number of different genera in a sample

was evaluated by bootstrap analysis, with resampling 1,000 times [27]. To construct the fungal phylogenetic trees, *Kluyveromyces lactis* NRRL Y-8279 NR_131273 was used as the outgroup [27]. To construct the bacterial phylogenetic trees, *Staphylothermus hellenicus* strain DSM 12710^T (NR 074532) was used as the outgroup.

Biodiversity assessment. Microbial diversity of insect vector skin from each geographical group was determined using various metrics (Table 2). Margalef's richness (Dmg) index was used to examine the fungal and bacterial biota from each geographical group [28]. Diversity at the genus level was revealed using Margalef's richness index [29], Menhinick's index [30], Shannon diversity (H') [31], and Simpson's index (1-D) [31].

RESULTS AND DISCUSSION

Fungal and bacterial identification and distribution. We isolated 13 fungal and 63 bacterial morphologically distinct strains from pure cultures obtained from the cutaneous surface of the host insect *B. agrestis* (Table 1, Supplementary Table 1). The fungal isolates belonged to 2 phyla, 5 classes, and 7 genera (Table 3, Fig. 2A and 2B);

Table 3. Taxonomy of isolates and their ratios in each geographical group

Sampling sites		Phylum	Class	Genera	Genera ratio (%)
Site 1	Fungal species	Ascomycota (5)	Eurotiomycetes (3)	*Aspergillus* (1)	3.4
				Penicillium (2)	6.9
			Dothideomycetes (2)	*Cladosporium* (2)	6.9
	Bacterial species	Proteobacteria (9)	Gamma proteobacteria (5)	*Stenotrophomonas* (1)	3.4
				Kosakonia (1)	3.4
				Enterobacter (3)	10.3
			Beta Proteobacteria (2)	*Achromobacter* (1)	3.4
				Variovorax (1)	3.4
			Alpha Proteobacteria (2)	*Ochrobactrum* (2)	6.9
		Actinobacteria (12)	Actinobacteria (12)	*Rhodococcus* (4)	13.8
				Microbacterium (3)	10.3
				Gordonia (1)	3.4
				Arthrobacter (4)	13.8
		Firmicutes (3)	Bacilli (3)	*Bacillus* (2)	6.9
				Paenibacillus (1)	3.4
Site 2	Fungal species	Ascomycota (3)	Leotiomycetes (1)	*Acremonium* (1)	3.4
			Eurotiomycetes (1)	*Aspergillus* (1)	3.4
			Dothideomycetes (1)	*Cladosporium* (1)	3.4
	Bacterial species	Proteobacteria (8)	Gamma proteobacteria (6)	*Pseudomonas* (5)	17.2
				Leclercia (1)	3.4
			Alpha proteobacteria (1)	*Rhizobium* (1)	3.4
			Beta Proteobacteria (1)	*Variovorax* (1)	3.4
		Actinobacteria (5)	Actinobacteria (5)	*Microbacterium* (2)	6.9
				Cellulosimicrobium (1)	3.4
				Arthrobacter (1)	3.4
				Rhodococcus (1)	3.4
		Firmicutes (7)	Bacilli (7)	*Bacillus* (7)	24.1
		Bacteroidetes (6)	Flavobacteria (6)	*Chryseobacterium* (6)	20.7
Site 3	Fungal species	Ascomycota (4)	Dothideomycetes (2)	*Cladosporium* (2)	11.1
			Sordariomycetes (2)	*Ilyonectria* (1)	5.6
				Tolypocladium (1)	5.6
		Basidiomycota (1)	Tremellomycetes (1)	*Trichosporon* (1)	5.6
	Bacterial species	Proteobacteria (6)	Gamma proteobacteria (4)	*Acinetobacter* (1)	5.6
				Serratia (2)	11.1
				Enterobacter (1)	5.6
			Alpha Proteobacteria (3)	*Ochrobactrum* (2)	11.1
				Novosphingobium (1)	5.6
			Beta proteobacteria (1)	*Delftia* (1)	5.6
		Actinobacteria (3)	Actinobacteria (3)	*Leifsonia* (1)	5.6
				Leucobacter (1)	5.6
				Rhodococcus (1)	5.6
		Firmicutes (2)	Bacilli (2)	*Bacillus* (2)	11.1

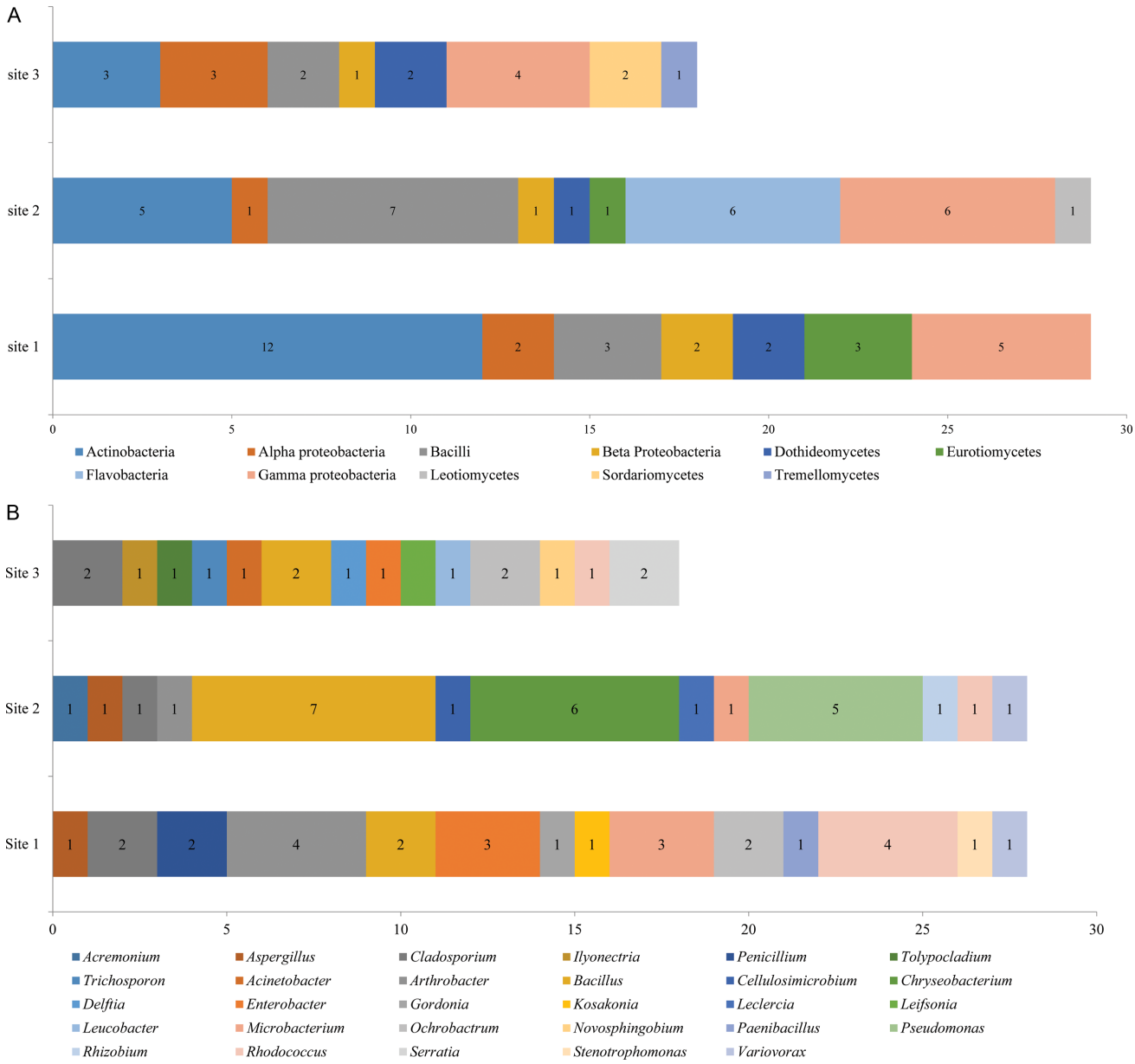


Fig. 2. Distribution of microbial isolates in each geographical vector group. Distributions are based on the class level (A) and genus level (B).

bacterial species belonged to 23 genera and 32 species (Table 3, Fig. 3A and 3B). On the basis of identification and phylogenetic analysis of the fungal strains (Fig. 3A–3C), most of the isolates belonged to the phylum Ascomycota, excluding a single fungal isolate that belongs to Basidomycetes (genus *Trichosporon* in geographical region 3). The most prevalent fungal genus was *Cladosporium* (38.5%), which was identified in all three geographical vector populations. According to bacterial identification and phylogeny (Fig. 4A–4C), the isolates were determined to belong to four phyla. The predominant group was Proteobacteria (39.7%), followed by Actinobacteria (31.7%), Firmicutes (19.1%), and Bacteroidetes (9.5%) (Table 3). In the proteobacterial group, Gammaproteobacteria was the predominant class (60.0%), and the most prevalent genera were *Bacillus* (17.5%), *Chryseobacterium* (9.5%), *Microbacterium* (9.5%), *Rhodococcus* (9.5%), *Pseudomonas* (7.9%), and *Arthrobacter* (7.9%). More than 20% of total isolates showed the potential to be novel bacterial species based on a sequence similarity less than 98.5% to existing strain types found in the EzTaxon or BLAST database [23].

Comparison of the results from each geographical group showed that the distribution or constituents of insect flora varied according to vector habitat (Table 3). At site 1, *Cladosporium* and *Penicillium* were the predominant fungal species, whereas *Rhodococcus*, *Arthrobacter*, *Microbacterium*, and *Enterobacter* were the predominant bacterial species. At site 2, however, *Bacillus* (28.0%), *Chryseobacterium*

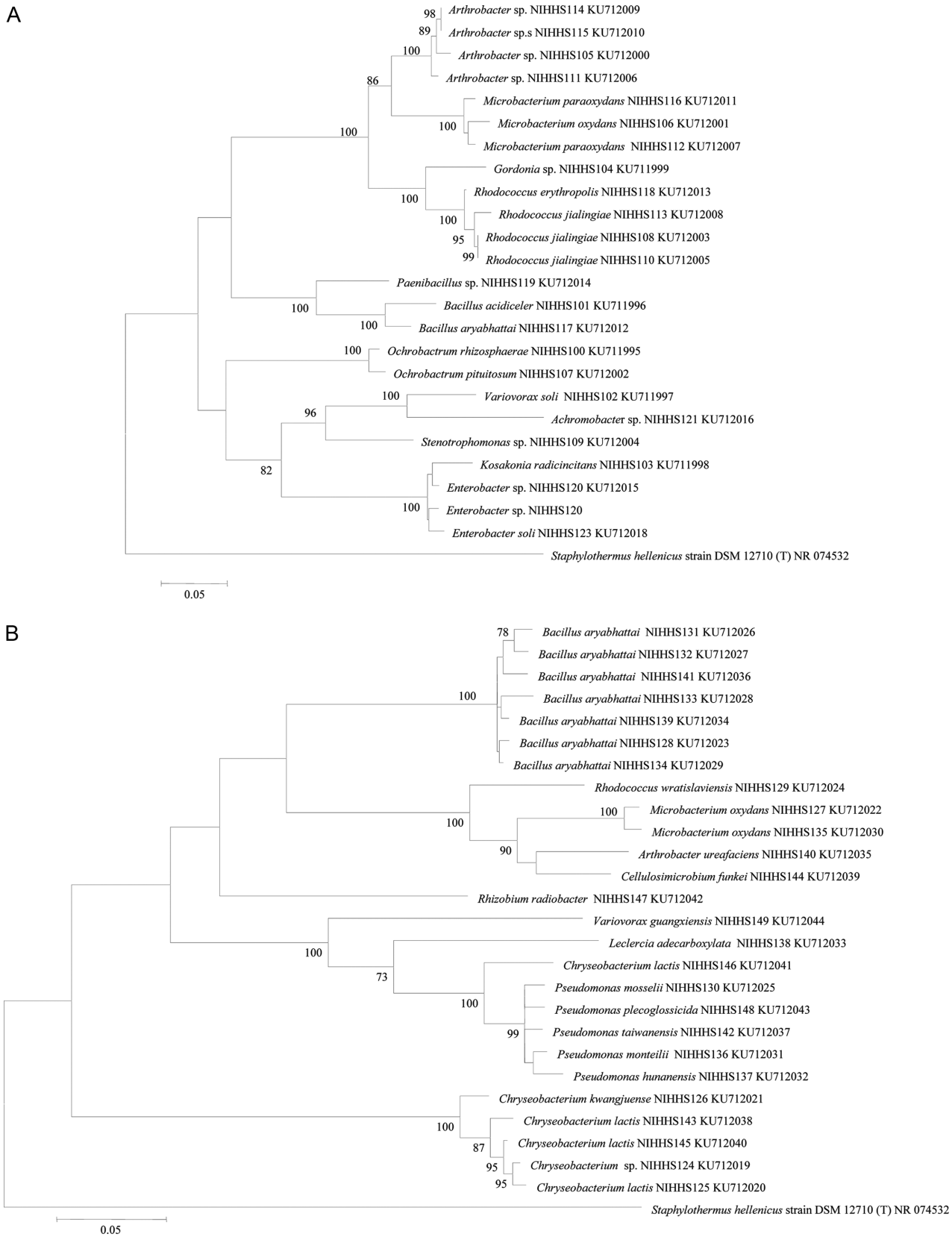
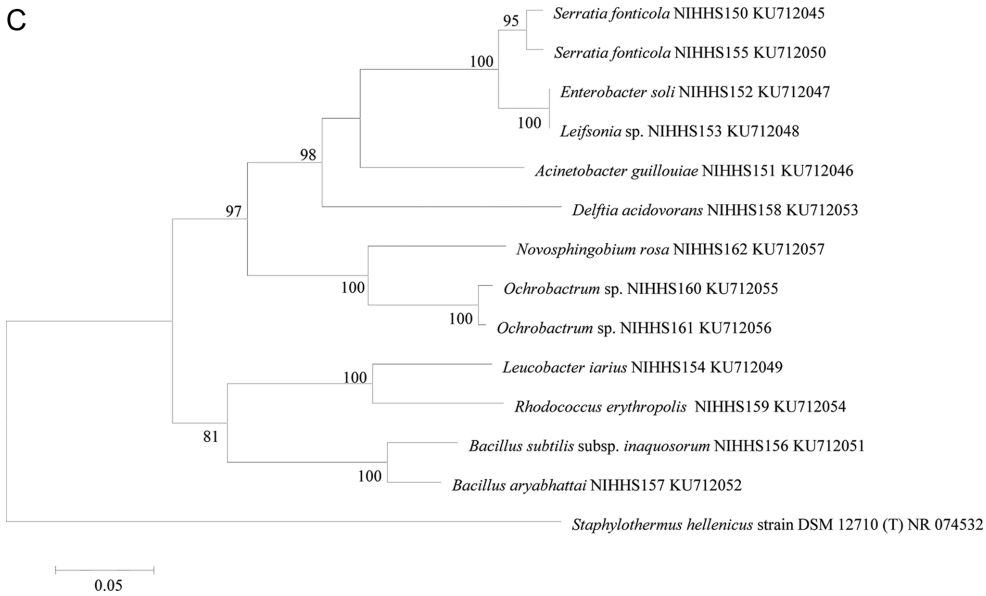


Fig. 3. Phylogenetic trees of fungal flora from each geographic vector group. Trees were constructed using fungal internal transcribed spacer sequences. Trees of isolates from site 1 (A), site 2 (B), and site 3 (C) were obtained using the maximum-likelihood (ML) algorithm with the Kimura 2-parameters. To construct the fungal phylogenetic trees, *Kluyveromyces lactis* NRRL Y-8279 NR_131273 was used as the outgroup, and the stability of relationships was evaluated by bootstrap analysis, with resampling 1,000 times.



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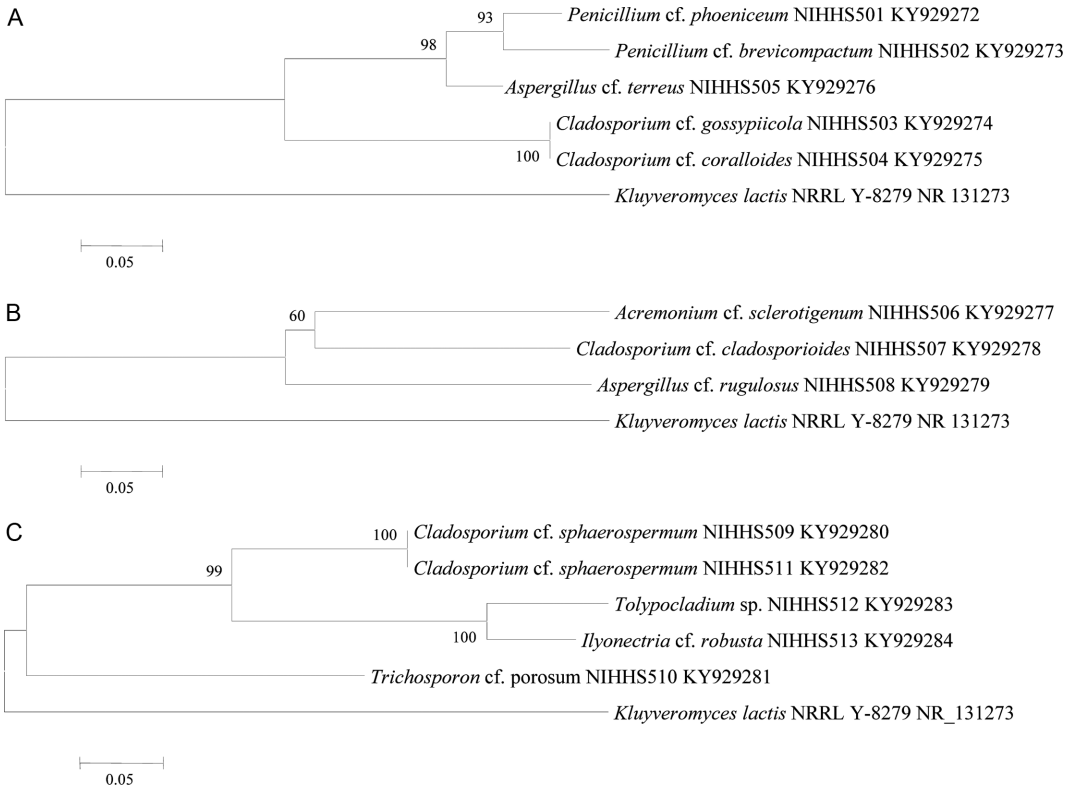

Fig. 4. Phylogenetic trees of bacterial flora from each geographic vector group. Trees were constructed using bacterial 16S rRNA genes. Trees of isolates from site 1 (A), site 2 (B), and site 3 (C) are shown. To construct the bacterial phylogenetic trees, *Staphylothermus hellenicus* strain DSM 12710^{T} (NR 074532) was used as the outgroup.

(24.0%), and *Pseudomonas* (20.0%) were the dominant bacterial species, but there was no specific dominant fungal species. At site 3, *Cladosporium* was the dominant fungal group, and *Ochrobactrum*, *Bacillus*, and *Serratia* were the dominating bacterial species. Fungal species from vector cuticles have often been regarded as useful resources for bio-control. Fungal species compatible with their host vector can survive in the environment for months as spores. Therefore, comparative analysis of cutaneous fungal flora will provide fundamental information [32] for developing

novel bio-control strategies aimed at combating the emerging *B. agrestis* problem.

Many fungal genera showing high homology with the isolates were reported to be crop pathogens based on their taxonomy. Some species of *Cladosporium* (identified at sites 1, 2, and 3), *Aspergillus* (identified at sites 1 and 2), and *Ilyonectria* (identified at site 3) are known as ubiquitous environmental saprobic or endophytic fungi, as well as major plant pathogens [33-36]. In contrast to these potentially harmful pathogens, some beneficial fungal species were also identified in this study. *Trichosporon* species showed the closest homology with isolates from site 3, suggesting their potential for antagonistic and antibiotic effects against pathogenic microorganisms of plants [37]. *Aspergillus terreus*, which showed high homology with fungal isolates in site 1, is a microorganism that is beneficial to its host plant, as a constituent of the endophytic fungal community, and is not a phytopathogen. Furthermore, *A. terreus* is a close symbiont of some other insects such as dragonflies [38]. This fungal taxon exhibits antifungal activity comparable to that of existing chemical antifungal agents against the growth of *Alternaria solani* and *Fusarium oxysporum* [38]. In general, confirmation of specific microbial taxa is not possible using fungal ITS profiling alone, and thus further taxonomic analysis is needed to secure candidates for controlling harmful insect vectors. Obtaining detailed taxonomic information on these fungal flora will provide fundamental information to develop effective bio-control strategies aimed at emerging problems such as *B. agrestis*. Meanwhile, *Kosakonia radicincitans* [39, 40], *Rhodococcus erythropolis* [41], and *Serratia fonticola* [42, 43] have been reported to have beneficial effects for crop plants or agricultural environments. Further studies will be required to determine the taxonomic relationships of these isolates.

Microbial diversity. The Shannon's diversity (H′) index of the three sites was similar (2.50, 2.17, and 2.58) (Table 4). Furthermore, Margalef's richness index (Dmg) (3.90, 3.60, and 4.50) and Menhinick's index (Dmn) (2.65, 2.46, and 3.30) showed differences between sites: site 3 had the highest values among all sites. Notably, site 3 had the lowest value (0.01) for Simpson's index (D), which likely reflects species dominance in the community. These contrasting results may be due to the lower abundance of certain genera at site 3 compared to those at site 1 (*Arthrobacter* or *Rhodococcus*) or site 2 (*Bacillus*, *Chryseobacterium, Pseudomonas*), which may have been influenced by local habitat and environmental factors. For these genera, there were over four species identified for a given genus. However, no isolates at site 3 showed a particularly large abundance for a given genus, although the total genera numbers were similar to those at site 1 or site 2.

Table 4. Microbial diversity of each geographical group

Microbial taxon	Site 1	Site 2	Site 3
Total isolates number	28	28	18
S (number of genera)	14	13	14
Shannon's index (H′)	2.50	2.17	2.58
Margalef's richness (Dmg)	3.90	3.60	4.50
Menhinick's index (Dmn)	2.65	2.46	3.30
Simpson's index (D)	0.06	0.12	0.01

Symbionts: *Cladosporium, Bacillus*, and *Rhodococcus*. Regardless of the variance in the distributions of bacterial and fungal species among geographical vector groups, *Cladosporium, Bacillus*, and *Rhodococcus* were identified in all groups (Table 5). Identification of common microbial genera or species from different vector groups suggests close symbiotic relationships (symbiosis) with the host vector [44]. Therefore, comparison of insect microbial flora from geographically segregated (due to geographical barriers) groups provides important information regarding insect vector or fungal disease control. These results were based on the principles of co-evolutionary relationship that commonly identified microbial flora identified in a specific host from ecologically segregated regions can represent a relatively strong interdependency of a host-microbe

Table 5. Microbial distribution per geographical group

Genera	Site 1	Site 2	Site 3
Acremonium	-	1	-
Aspergillus	1	1	-
Cladosporium	2	1	2
Ilyonectria	-	-	1
Penicillium	2	-	-
Tolypocladium	-	-	1
Trichosporon	-	-	1
Acinetobacter	-	-	1
Arthrobacter	4	1	-
Bacillus	2	7	2
Cellulosimicrobium	-	1	-
Chryseobacterium	-	6	-
Delftia	-	-	1
Enterobacter	3	-	1
Gordonia	1	-	-
Kosakonia	1	-	-
Leclercia	-	1	-
Leifsonia	-	-	1
Leucobacter	-	-	1
Microbacterium	3	1	-
Ochrobactrum	2	-	2
Novosphingobium	-	-	1
Paenibacillus	1	-	-
Pseudomonas	-	5	-
Rhizobium	-	1	-
Rhodococcus	4	1	1
Serratia	-	-	2
Stenotrophomonas	1	-	-
Variovorax	1	1	-

The numbers indicate the number of identified species belonging to each genus.

interaction. For example, if certain species of *Lactobacillus*, as human gastrointestinal commensal flora, are identified in all human ethnic groups, including different races or geographically segregated populations (such as those residing on physically separated islands or mountains), it can be concluded that *Lactobacillus* species have a close evolutionary association with humans. Therefore, this species can be a good candidate to serve as a stable drug deliverer of effective molecules such as insulin genes, and for the production of insulin hormones given their ability for successful colonization and settlement in the gut of all human populations. By this same logic, identification of commonly identified microbial strains from geographically segregated hosts is an important indicator of interdependency with their host. By contrast, if a given microbial species is identified in only one vector population and not in another that is ecologically or physically separated, strong interdependencies between the host vector and microbe cannot be inferred. In the field of biological control, strategies for controlling specific insect vector (such as cockroach or fly) populations may not result in complete eradication of the associated microbe, as in the case of *Salmonella* or *Shigella* species, which do not show strong interdependency to their host vector, given that these bacteria can also be isolated from other diverse insect vectors.

Microbes interacting with their host vector could have positive or negative effects within the outbreak locale. If they are plant pathogens that form close symbiotic relationships with the vector, they could be a good control target to prevent fungal disease manifestation in agricultural lands. However, microbes that form positive symbiotic relationships with the agricultural environment could be developed or proposed as good biological control mediators [15, 45]. For example, *Bacillus aryabhattai*, which was identified as a common species in this study, may be a close symbiont of *B. agrestis* (Tables 5 and 6). Most species belonging to the *Bacillus* genus, especially *B. aryabhattai*, are not plant pathogens. Instead, they confer diverse beneficial effects on plant growth, such as inducing plant resistance to rhizosphere conditions and reducing disease incidence in agricultural soils [46-49]. Therefore, *Bacillus*, especially *B. aryabhattai*, may exert beneficial roles via the secretion of effector molecules such as anti-parasitic antibodies [15, 50-52]. However, the commonly distributed fungal *Cladosporium*

Table 6. Bacterial species distribution in each geographical group

Genera	Species	Site 1	Site 2	Site 3
Acinetobacter	*guillouiae*	-	-	1
Arthrobacter	*pascens*	1	-	-
	ureafaciens	-	1	-
Bacillus	*acidiceler*	1	-	-
	aryabhattai	1	7	1
	subtilis subsp. *inaquosorum*	-	-	1
Cellulosimicrobium	*funkei*	-	1	-
Chryseobacterium	*kwangjuense*	-	1	-
	lactis	-	4	-
Delftia	*acidovorans*	-	-	1
Enterobacter	*soli*	2	-	1
Gordonia	*effusa*	-	-	-
Kosakonia	*radicincitans*	1	-	-
Leclercia	*adecarboxylata*	-	1	-
Leucobacter	*iarius*	-	-	1
Microbacterium	*oxydans*	1	2	-
	paraoxydans	2	-	-
Ochrobactrum	*pituitosum*	1	-	-
	rhizosphaerae	1	-	-
Novosphingobium	*rosa*	-	-	1
Pseudomonas	*hunanensis*	-	1	-
	monteilii	-	1	-
	mosselii	-	1	-
	plecoglossicida	-	1	-
	taiwanensis	-	1	-
Rhizobium	*radiobacter*	-	1	-
Rhodococcus	*erythropolis*	1	-	1
	jialingiae	3	-	-
	wratislaviensis	-	1	-
Serratia	*fonticola*	-	-	2
Variovorax	*guangxiensis*	-	1	-
	soli	1	-	-

species found in this study have been reported to have potential for plant pathogenicity [38, 53]. Therefore, these fungal species could be critical in controlling pathogenic outbreaks, and thus further pathogenicity studies (i.e., disease-causing assays) in *Cladosporium* are required. So far, these species have not been reported as being transmissible by the insect vector *B. agrestis* [47].

In contrast to *Cladosporium* or *Bacillus* species, the region-dependent fungal (*Acremonium*, *Aspergillus*, *Ilyonectria*, *Penicillium*, *Tolypocladium*, and *Trichosporon*) and bacterial (*Pseudomonas*) genera could be classified as loosely associated symbionts, which demonstrate weak interdependent relationships with host vectors (e.g., synergism, protocooperation, and amensalism) [54]. These genera are not significantly dependent on the specific properties of host insects, and could establish relationships with other host species (known as 'host changes'). It may be interesting to note that the major determinants of the high Simpson index value (reflecting the abundance of specific genera) at site 2 were *Chryseobacterium* and *Pseudomonas*, which were not found in other sites. This suggests that strains that interact weakly with *B. agrestis* might be more strongly affected by environmental conditions than host vector characteristics.

Some *Pseudomonas* species (Table 6) have been reported to be representative plant pathogens [55-58]. In general, confirmation of disease-causing factors via 16S rDNA profiling is impossible. However, interactive relationships between the microflora and host vectors are determined by physiological interdependency [59], and species belonging to the same bacterial genera share comparatively similar physiological phenotypes. Therefore, if specific species are identified in the vector, another bacterial species in same genus could be part of the microflora in the same host vector [54].

Another possible issue worthy of consideration is the aforementioned phenomenon of host changes. This concept has been well-studied in the context of insect vectors that transmit human infectious disease. Causative agents such as *Flavivirus*, *Plasmodium*, and *Trypanosoma* are transferred only by specific insect vectors, including *Aedes*, *Anopheles*, and *Glossinidae* [4, 6, 52, 60, 61], and are known to exhibit strong host specificity. These host vectors provide optimal conditions for biological mechanical transmission, and could not be substituted with other vectors owing to strong dependency on their specific host vector. By contrast, species such as *Salmonella* and *Shigella* that do not show high interdependency with their host vector can readily change their insect host and be transferred by diverse biological vectors [3, 62]. On the basis of our results, the prominent bacterial genus in this study was *Pseudomonas*, which exhibits a non-specific host requirement. *Pseudomonas* is considered to be transferred to host plants by diverse host vectors [63, 64]. Climate change, and particularly global warming, can lead to the emergence of diverse insect vectors, which may cause severe economic damage by inducing changes in the hosts of potential plant pathogens.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary data including one table can be found with this article online at http://www.mycobiology.or.kr/src/sm/mb-45-160-s001.pdf.

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