

RESEARCH ARTICLE

Abundance and diversity of gut-symbiotic bacteria, the genus *Burkholderia* in overwintering *Riptortus pedestris* (Hemiptera: Alydidae) populations and soil in South Korea

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Abstract

Riptortus pedestris is a major agricultural pest on leguminous plants in South Korea and Japan. Recent studies have revealed that *R. pedestris* can form beneficial symbiosis with bacteria belonging to genus *Burkholderia* acquired from soil newly for every generation. Although their physiological interactions are relatively well-understood, infection rate and abundance of the *Burkholderia* in overwintering natural populations of *R. pedestris* remain unknown. Therefore, the objective of this study was to characterize *Burkholderia* infection ratio and clade composition of overwintering *R. pedestris* populations as well as prevalence and diversity of the genus *Burkholderia* in soil by conducting a two-year field survey. From the field survey, we found 29 overwintering *R. pedestris* adults in forested areas nearby soybean fields. Diagnostic PCR analysis revealed that overall infection rate of the symbiotic *Burkholderia* was 93.1% from overwintering adults. Among the *Burkholderia*-infected *R. pedestris*, 70.4% of individuals harbored unclassified *Burkholderia* clades whereas 22.2% and 7.4% of *R. pedestris* harbor stinkbug-associated beneficial and environmental (SBE) group and *Burkholderia cepacia* and complex (BCC), respectively. All *R. pedestris* were infected with a single clade of *Burkholderia*. In soil, 56.2% of soil samples were *Burkholderia* positive, and unlike *R. pedestris*, multiple *Burkholderia* clades were detected from 62.2% of those samples. Clade composition of the genus *Burkholderia* in the samples with the bacteria was 91.1%, 60.0%, 31.1% and 8.8% for plant-associated beneficial and environment (PBE), BCC, SBE and unclassified clade, respectively.

Introduction

During winter season, insects face thermally stressful environments. They have developed various strategies to successfully withstand and survive inhospitable environments through evolutionary interactions with organisms and abiotic conditions [1,2]. Their physiological strategies for cold hardness can be classified into two groups: freeze tolerant (synthesis of ice nucleating agents) and freeze avoidance (removal of all potential nucleates) [2–5]. In addition, insects can

behaviorally avoid inhospitable environments through long or short distance movement to less stressful conditions [2,6,7].

Understanding overwintering ecology is particularly important for pest species because levels of survived winter populations can serve as a barometer for estimating pest pressures in succeeding active seasons [1,3,4]. Indeed, there have been attempts to better manage pest populations by estimating overwintering population levels. For examples, the timing and size of spring emerge populations have been forecasted for aphid management by evaluating overwintering eggs of black bean aphid, *Aphis fabae* Scopoli (Hemiptera: Aphididae) [1,8,9] and cereal aphid, *Rhopalosiphum padi* Linnaeus (Hemiptera: Aphididae) [10].

Although overall density levels of overwintering pest populations are undoubtedly foundational information to forecast pest pressures in the spring, biological variations at individual level are also worthwhile to consider because these variances could affect the survival rate of overwintering insects themselves as well as their performance after emergence [1,11]. Physiological status and fitness of overwintering individuals are affected by various abiotic and biotic factors. Among them, symbiotic associations with microorganisms can play a pivotal role by making arthropod hosts more tolerant to thermally stressful environments [12–15]. Furthermore, it has been reported that symbiotic microbes can also enhance individual fitness of host arthropods under cold conditions [16].

The bean bug, *Riptortus pedestris* (Fabricius) (Hemiptera: Alydidae), is a polyphagous insect pest in East Asia including South Korea, Japan, and Taiwan [17–19]. Both adult and immature stages of *R. pedestris* attack leguminous plants, especially soybeans [17,20–22]. Recently, it has been revealed that *R. pedestris* possess symbiotic bacteria of the genus *Burkholderia* in the mid-gut crypts [23,24]. Interestingly, unlike typical insect-microbe symbiosis, *Burkholderia* are not transmitted vertically from parents to offspring. Instead, *R. pedestris* acquire their symbionts newly at every generation from soil environments mainly during the 2nd instar period [24–26]. Forming an insect-microbe symbiosis, symbionts are known to provide a series of enhancement of host fitness, including increased fecundity, decreased developmental period, and increased body size [24,25,27].

Although the effect of *Burkholderia* infection on the biology of active, non-diapausing *R. pedestris* is relatively well understood, interaction between symbionts and overwintering *R. pedestris* remains unknown. It is likely that *R. pedestris* with *Burkholderia* would impose a higher risk level against pest management compared to aposymbiotic individuals because symbiotic individuals are known to exhibit enhanced life history parameters such as fecundity and developmental rate. Moreover, Kikuchi et al. [28] have found that when *R. pedestris* acquire fenitrothion-degrading *Burkholderia* strain, these insects can have resistance to fenitrothion, a widely used insecticide against *R. pedestris*. Therefore, the infection rate of *Burkholderia* in overwintering *R. pedestris* population could serve as a basic information to accurately assess the risk level of overwintering population to crops in the spring.

Riptortus pedestris are known to enter facultative diapause, as opposed to preprogrammed obligate diapause, in adult stage with response to abiotic changes especially for short-day photoperiods [29–31]. In addition, a recent study has revealed that adult *R. pedestris* can use leaf litter as a primary overwintering structure and that overwintering individuals are randomly distributed when forested areas adjacent to crop fields are surveyed [32]. The objective of the present study was to determine infection rate and clade composition of *Burkholderia* in overwintering *R. pedestris* and prevalence and diversity of the genus *Burkholderia* in soil environments by conducting a two-year field survey in South Korea. We collected leaf litter from forested areas nearby soybean fields from eight provinces in South Korea to locate overwintering *R. pedestris*. We also collected soil samples at the same locations from which leaf litter were collected. All overwintering *R. pedestris* found and soil samples collected were individually

subjected to molecular analysis to assess the prevalence of *Burkholderia* in both insects and soil samples.

Materials and methods

Field survey

A field survey was conducted over two winter seasons from January to February in 2017 and 2018 to characterize infection rate and clade composition of genus *Burkholderia* in overwintering *R. pedestris* populations as well as prevalence and diversity of *Burkholderia* in soil samples of South Korea. For the survey, a representative soybean field was selected in each province in South Korea based on the Korean Statistical Information Service (KOSIS) [33]: Paju (37° 57'22.06"N 126° 52'7.53"E), Inje (37° 57'57.61"N 128° 17'35.09"E), Goesan (36° 53'36.91"N 127° 49'22.61"E), Gongju (36° 17'3.14"N 127° 2'25.65"E), Andong (36° 36'45.12"N 128° 37'1.22"E), Miryang (35° 23'6.74"N 128° 47'7.99"E), Gochang (35° 25'50.50"N 126° 37'37.46"E), and Muan (34° 59'43.24"N 126° 27'21.62"E) (Fig 1). In each province, a sampling site was selected in the forested area located within ca. 1 km from the soybean field. In each sampling site, five sampling points were established with a distance of ca. 20 m apart from each other located at similar elevations. At each sampling point, a 1 × 1 m quadrat was drawn. Then all organic materials over the soil layer mainly consisting of leaf litter were carefully collected into a plastic bag [34]. After collecting the leaf litter sample, 30 ml of soil sample was also collected and sealed in a 50 ml conical tube (Hyundai micro Co., Ltd., Seoul, South Korea).

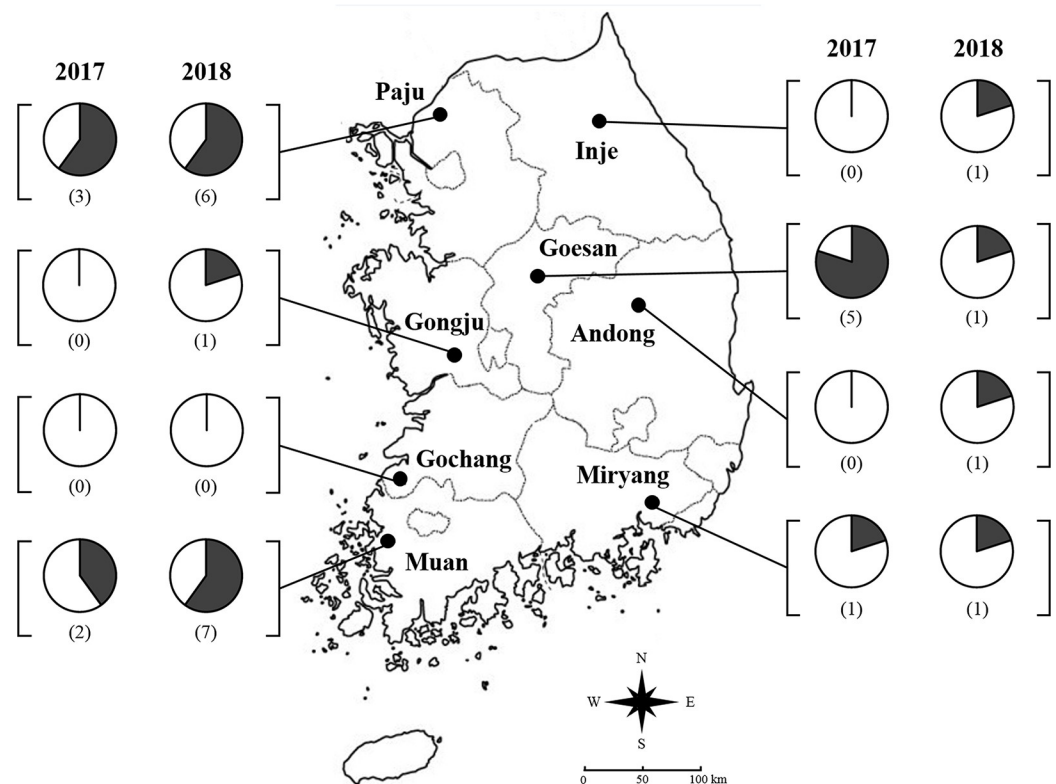


Fig 1. Geographical locations of eight sampling sites in South Korea from the two-year field survey. Pie chart indicate the ratio of leaf litter samples in which overwintering *Riptortus pedestris* found from 2017 and 2018, respectively. Parentheses present below the pie chart show the number of overwintering *R. pedestris* found in leaf litter samples collected.

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Sample processing

Collected leaf litter samples were brought to the laboratory and kept in an indoor area (temperature ca. 4°C) until samples were examined to locate overwintering *R. pedestris*. When live overwintering *R. pedestris* or other live overwintering hemipteran species were detected, insects were individually kept in 1.5-ml conical tubes (Axygen Biosciences, Hangzhou, China) with 90% ethanol after species and sex identification. Dominant leaf types in sampling areas were confirmed based on Forest Geospatial Information System (FGIS) [34]. After sample inspection, leaf litter samples were completely dried in a dry-oven (BF-150C, Biofree Co. Ltd., Seoul, South Korea) at 80°C for 72 hours to estimate moisture contents.

DNA extraction

Collected overwintering *R. pedestris* and soil samples were individually subjected to DNA extraction and diagnostic PCR. The abdomen part of *R. pedestris* was used for DNA extraction using MagListo™ 5M Genomic DNA Extraction Kit (Bioneer Co. Ltd., Daejeon, South Korea) according to the manufacturer’s instructions. DNA extractions for soil samples were performed with 25 mg of soil per sampling point using DNeasy PowerSoil kit (QIAGEN, Hilden, Germany).

Diagnostic PCR

PCR analysis was conducted to determine the presence of specific *Burkholderia* clades in total DNA extracts from insect and soil samples. First, all samples were subjected to PCR analysis to detect the presence of genus *Burkholderia* using *Burkholderia*-specific 16s rRNA primers (Table 1).

Then, *Burkholderia* positive samples were subjected to subsequent PCR analysis to determine the *Burkholderia* clade composition in the samples using clade-specific primer sets including BCC, PBE, and SBE clades (Table 1) with temperature profile of 94°C for 5 min followed by 35 cycles of 94°C for 30s, 55°C for 1 min, and 72°C for 2 min [26,35]. Gel electrophoresis was performed to confirm the presence of target primers on a 1.0% agarose gel slab containing Tris-acetate-EDTA buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.3) at 120 v/cm (Mupid-exU, Takara Bio Inc., Shiga, Japan) followed by visualization on a digital gel

Table 1. Primers and probes used in this study.

Target group	Target gene	Primer/Probe name	Nucleotide sequence (5' → 3')	Approximate product size (kb)	Annealing temp (°C)	Reference
<i>Burkholderia</i>						
<i>Burkholderia</i> spp.	16s rRNA	Burk16SF	TTTTGGACAATGGGGGCAAC	0.7	55	Kikuchi et al. [23]
		Burk16SR	GCTCTTGCGTAGCAACTAAG			
PBE clade	16S rRNA	Burk16SF	TTTTGGACAATGGGGGCAAC	0.5	55	Itoh et al. [35]
		PBE822R	CTTCGTTACCAAGTCAATGAAGA			
BCC clade	16S rRNA	BCC370F	TTTTGGACAATGGGCGAAAG	0.8	55	Itoh et al. [35]
		Burk16SR	GCTCTTGCGTAGCAACTAAG			
SBE clade	16S rRNA	SBE160F	CGCATACGACCTAAGGGA	1.3	55	Itoh et al. [35]
		SBE1400R	CTTGC GGTTAGGCTACCT			
<i>R. pedestris</i>	COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	0.7	48	Folmer et al. [36]
		HCO2198	TAAACTTCAGGGTGACCAAAAAATCA			

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documentation system. *Burkholderia* positive samples that did not belong to any of the three clades were defined as unclassified in this study.

Results

Overwintering *R. pedestris* populations

From the two-year field survey, a total of 11 and 18 overwintering *R. pedestris* adults were collected from 2017 and 2018, respectively (Fig 1 and S1 Table). Among the eight provinces surveyed, no overwintering *R. pedestris* was found in Gochang over the two-year survey whereas overwintering individuals were found from Paju, Muan, Goesan, and Miryang in both years. The greatest number (a total of 9 individuals found per province) of overwintering *R. pedestris* was found in Paju and Muan over two years. Percentages of leaf litter samples containing *R. pedestris* ranged from 0 to 80% in both 2017 and 2018 across provinces surveyed. In general, overwintering *R. pedestris* were present solitarily in leaf litter samples (76.2% of leaf litters contained a single individual). Over the two-year survey, a total of 12 males and 17 females were found. The sex ratio was not significantly deviated from 1:1 (Likelihood Ratio = 0.4415, $P = 0.5061$). In addition, a total of 22 hemipteran species in families of Coreidae and Pentatomidae were collected over two years, including *Cletus punctiger* Dallas (Hemiptera: Coreidae), winter cherry bug, *Acanthocoris sordidus* Thunberg (Hemiptera: Coreidae), and brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae).

Characteristics of leaf litter samples were compared between the two groups with and without overwintering *R. pedestris* found (Fig 2). There was no significant difference in the altitude of leaf litter location between the two groups ($t = 0.75$, $P = 0.4556$). There was no significant difference in depth ($t = 0.20$, $P = 0.8348$) or moisture content ($t = 0.94$, $P = 0.3512$) between *R. pedestris*-detected and non-detected samples collected either. Based on FGIS [34], Overwintering *R. pedestris* were almost exclusively detected from deciduous leaf leaves especially from Family Fagaceae whereas only one individual was found from a forest mainly with Family Pinaceae.

Characteristics of *Burkholderia* in overwintering *R. pedestris*

Among 29 overwintering *R. pedestris* collected over two years, 93.1% of individuals were infected with genus *Burkholderia* (S1 Table). Only two individuals found in 2018 were not infected with these bacteria (Fig 3). In *Burkholderia*-infected *R. pedestris*, 70.3% of individuals harbored unclassified *Burkholderia* strains whereas 22.2 and 7.4% of *Burkholderia* found in *R.*

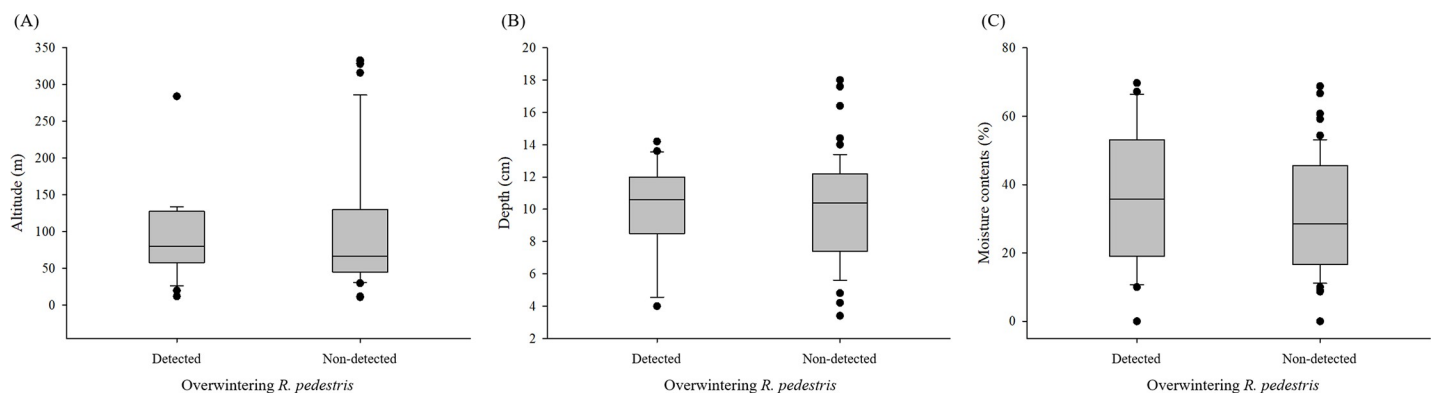


Fig 2. Characteristics of leaf litter samples with or without overwintering *Riptortus pedestris* found. (A) Altitude of leaf litter location, (B) Depth of leaf litter sample above the soil base layer, and (C) Moisture content of leaf litter sample.

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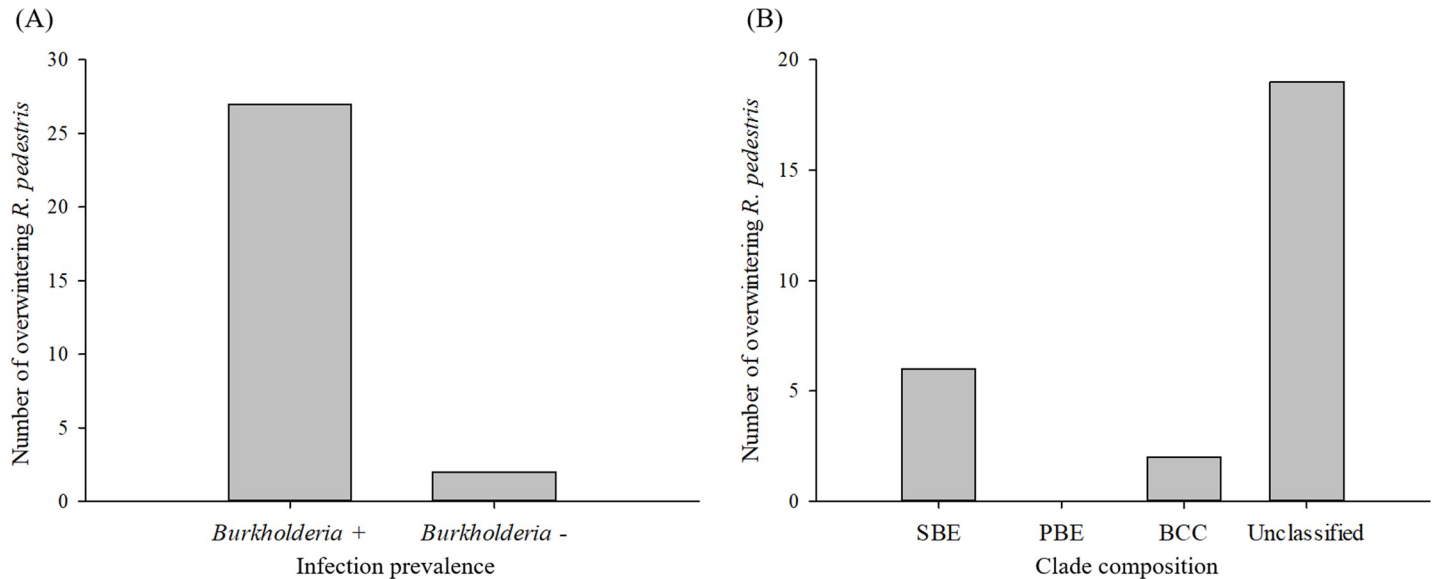


Fig 3. Characteristics of *Burkholderia* infection and clade composition in overwintering *Riptortus pedestris* found from leaf litter samples over the two-year survey. (A) Overall number of insects infected with genus *Burkholderia*, and (B) Clade composition of genus *Burkholderia* in insects.

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pedestris were identified as SBE and BCC clades, respectively (Fig 3). All infected *R. pedestris* individuals harbored a single *Burkholderia* clade without multiple strain infection.

Characteristics of *Burkholderia* in soil

Among 80 soil samples collected over two years, 56.2% of soil samples were *Burkholderia* positive (Fig 4 and S2 Table). In *Burkholderia* positive soil samples, 24.4% of soil samples were detected with all three clades in genus *Burkholderia* tested, whereas 31.1 and 6.7% of samples were detected with PBE + BCC clades and PBE + SBE clades, respectively (Fig 4). In addition, 28.9% samples were detected only with a single clade, PBE, while 8.9% samples were positive for unclassified *Burkholderia*. Overall, PBE clade was most frequently detected from soil samples. This clade was detected in 41 out of 55 *Burkholderia* positive soil samples (Fig 4).

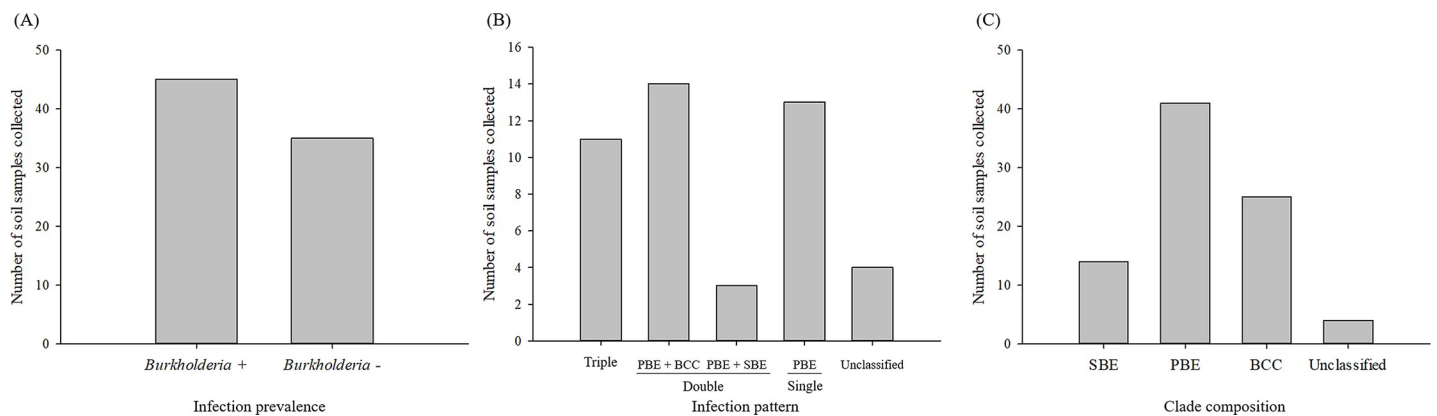


Fig 4. Characteristics of *Burkholderia* positive and clade composition in soil samples collected over the two-year survey. (A) Overall number of genus *Burkholderia* positive soil, (B) Clade composition of genus *Burkholderia* in the soil, and (C) Total frequency of *Burkholderia* clade detected from soil samples.

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Ethics statement

No specific permits were required for the described field survey.

Discussion

Infection rate and clade composition of symbiotic *Burkholderia* in *R. pedestris* field populations provides critical information to better design and implement pest management programs against this economically-important pest. Although it is currently unknown how symbionts affect the biology of overwintering *R. pedestris*, it is expected that higher infection rates in overwintering populations would impose higher risk to crops by survived pests in the spring. This is because symbiotic *Burkholderia* are known to provide a series of enhancement for *R. pedestris* fitness, including larger body size, increased fecundity, and enhanced insecticide resistance [24,25,28]. For this reason, this study investigated infection rate of *Burkholderia* in overwintering *R. pedestris* populations in nature for the first time. In this study, DNA sample was extracted from the whole abdomen of overwintering *R. pedestris*, which might have resulted in detection of *Burkholderia* from gut sections other than the crypt-bearing M4 region, where the symbiont is known to colonize in the insect. Nevertheless, overwintering individuals generally clear their gut lumen to prevent freeze damage because the particles in gut lumen can act as ice nucleators, reducing the chance of detecting the symbionts from other gut sections [2].

Results of this study indicate that overwintering adults of *R. pedestris* are infected with *Burkholderia* with a high rate, yielding 93.1% across the two-year survey. This high infection rate in overwintering adults is consistent with results of field study conducted by Kikuchi et al. [23] in Japan. They examined active, non-diapausing *R. pedestris* adults and found that the infection rate was as high as 97.8%. However, we should carefully interpret the high infection rate in overwintering populations because it is currently unknown whether *Burkholderia* infection affects searching behavior of *R. pedestris* adults for overwintering sites or their survivorship during the winter. That is, we cannot rule out the possibility that aposymbiotic individuals are less likely to successfully locate overwintering sites or may suffer from higher mortality in thermally stressful environments. Indeed, a recent study has revealed that a specific bacteria species in *Drosophila* microbiome is essential for the fly to exhibit coordinated locomotion behavior [37]. Moreover, the survival rate and mobility of black-legged tick are significantly enhanced when they form symbiosis with *Anaplasma phagocytophilum* [16].

With respect to clade composition of *Burkholderia* found in overwintering *R. pedestris*, SBE and BCC clades were detected from the abdomen of this insect. The infection rate with SBE clade known to specifically form associations with stink bugs [24] was 22.2% among overwintering individuals. Therefore, these insects with SBE might have enhanced fitness such as fecundity as reported in previous studies [24,25]. Interestingly, two overwintering *R. pedestris* harbored BCC clade in their body. BCC clade is known as a pathogen infecting human, animals, and plants without previous report of its association with *R. pedestris*. This result suggests that relationships of BCC clade with *R. pedestris* and potential effects of the clade on the biology of insects need to be investigated in the future. In addition, 70.3% of *Burkholderia* found in the overwintering *R. pedestris* were not phylogenetically grouped into any of the three clades previously classified. Thus, further studies should identify phylogenetic relationships of this unclassified *Burkholderia* group with other clades and its biological associations with *R. pedestris*.

At geographical scale of this study, the percentage of *Burkholderia* positive soil samples was substantially lower than that found from overwintering *R. pedestris*. Moreover, SBE clade was detected from only 17.5% of the samples. As somewhat expected, PBE clade known as a plant-associated beneficial and environmental group that could promote plant growth [24,38], was

the most prevalent group in soil, with PBE clade being detected from 51.3% of the soil samples. Although the proportion of *Burkholderia* positive samples in soil was slightly greater than 50%, it was notable that the soil samples detected with multiple *Burkholderia* clades were fairly common in *Burkholderia*-positive samples. These results indicate that *Burkholderia* are likely to be distributed in patch among soil environments, although multiple clades are present together in the patch. Nevertheless, soil microbiota tends to be complex with enormously diverse microorganisms and therefore our use of diagnostic PCR could have resulted in false-positives. For this reason, caution is needed when interpreting the soil sample results. Further analysis using sequencing or next generation sequencing may be warranted to identify and characterize their composition at a higher accuracy.

Our results revealed that all *R. pedestris* harboring *Burkholderia* were infected with a single clade. This strongly suggests that *R. pedestris* insects are capable of selectively colonizing their mid-gut with a single, specific *Burkholderia* clade. Indeed, Ohbayashi et al. [39] have demonstrated that *R. pedestris* insects have an intestinal organ known as constricted region to sort symbionts for selective colonization by *Burkholderia* in the mid-gut.

Consistent with results of a previous study [32], our two-year survey confirmed that adult *R. pedestris* could overwinter in the forested area adjacent to crop fields. This study also revealed that this insect species could utilize deciduous leaf litter in family Fagaceae as overwintering structures. However, there was no evidence supporting that the altitude of leaf litter location, the depth of leaf litter, or the moisture content of leaf litter could affect the likelihood of finding overwintering individuals. That is, it is not feasible with current results to recommend site-specific management tactics within the landscape scale of this study. Further studies should examine variations of micro climate conditions within deciduous forest and their potential effects on overwintering site selection by *R. pedestris*.

In summary, we characterized the infection rate and clade composition of *Burkholderia* in overwintering insect populations for the first time. In addition, we report the prevalence and clade composition of *Burkholderia* in soil which serves as a primary environment for *R. pedestris* to newly acquire symbionts for every generation. In most pest management systems, overwintering ecology of target species has been neglected due to difficulty of field survey [1]. Therefore, information reported here may serve as basis to improve pest management programs against *R. pedestris* by characterizing risk levels of pest populations based on the size of overwintering populations and their symbiont-associated fitness levels.

Supporting information

S1 Table. Infection prevalence and clade composition of the genus *Burkholderia* in the overwintering *R. pedestris* detected over 2-yr field survey.

(DOCX)

S2 Table. *Burkholderia* positive ratio and clade composition in soil samples collected over the two-year survey.

(DOCX)

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Author Contributions

Conceptualization: Minhyung Jung, Doo-Hyung Lee.

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