



Characterization of *Burkholderia* bacteria clade compositions in soil and *Riptortus pedestris* (Hemiptera: Alydidae) in South Korea

Joo-Young Kim, Minhyung Jung, Doo-Hyung Lee*

Department of Life Sciences, Gachon University, Gyeonggi-do, South Korea



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ABSTRACT

Riptortus pedestris (Hemiptera: Alydidae) is known to acquire the genus *Burkholderia*, symbiotic bacteria, from soil. Therefore, symbiont acquisition of *R. pedestris* would be directly affected by bacterial diversity in soil. Soil typically harbors diverse microbes including different *Burkholderia* clades such as SBE (stinkbug-associated beneficial and environmental), PBE (plant-associated beneficial and environmental), and BCC (*Burkholderia cepacia* and complex). Nevertheless, little is known about *Burkholderia* acquisition patterns of *R. pedestris* in nature, especially in the context of bacteria clade compositions in soil. Therefore, based on diagnostic PCR analysis, we investigated *Burkholderia* clade compositions in field-collected soil itself and *R. pedestris* when the insects were provided with the soil. Also, wild *R. pedestris* were surveyed to characterize their *Burkholderia* compositions. First, 88.44% of soil samples were detected with the genus *Burkholderia*, and triple clade (SBE + PBE + BCC) was most frequently detected. Second, *R. pedestris* nymphs readily acquired *Burkholderia* bacteria from field-collected soil where 91.25% of the reared insects harbored the bacteria in their midguts. In contrast to soil, the detection of single BCC clade was the most dominant among the three identified *Burkholderia* clades. Third, from wild *R. pedestris*, 80.62% of the insects were found to harbor the genus *Burkholderia*, and single BCC clade was most frequently detected. Finally, 29.13% and 47.06% of the reared and wild *R. pedestris* were detected with unidentified *Burkholderia* clade, which does not belong to any of the three identified clades. Our findings provide baseline information to better understand ecological associations between *R. pedestris* and *Burkholderia* bacteria in different clades.

Introduction

Insects, the most abundant animal group, are known to establish important associations with bacterial symbionts in nature (Bennett and Moran, 2015; Engel and Moran, 2013; Ferrari and Vavre, 2011). These symbionts provide a variety of benefits to their hosts such as aiding the digestive process (Baumann, 2005), providing essential nutrients (Douglas, 1998), and synthesizing defense materials against parasites (Vorburger, 2014). To maintain and transfer beneficial symbionts from parents to their offspring, insects in general have developed two different transmission strategies: intracellular and extracellular transmissions (McClure et al., 2017; Salem et al., 2015). With intracellular transmission, symbionts are transferred from parents to offspring during oogenesis or embryogenesis (e.g., aphids - *Buchnera* or tsetse flies - *Wigglesworthia*) (Koga et al., 2012; Rio et al., 2012). By contrast, with extracellular transmission, symbionts are transmitted outside of the hosts using various transmission routes such as egg smearing

(*Pyrrhocoris apterus* - *Coriobacterium glomerans*) (Salem et al., 2013), capsule transmission (*Megacopta punctatissima* - *Candidatus Ishikawaella capsulata*) (Hosokawa et al., 2005; Nikoh et al., 2011), and jelly transmission (*Parastrachia japonensis* - *Candidatus Benitsuchiphilus tojoi*) (Hosokawa et al., 2012).

In contrast to these vertical transmissions described above, recent studies have revealed that several hemipteran species can acquire their symbionts from the environment such as plants and soil (i.e., environmental acquisition) (Caspi-Fluger et al., 2011). Symbiosis between *Riptortus pedestris* (Hemiptera: Alydidae) and *Burkholderia* bacteria is a representative example of environmental acquisition (Kikuchi et al., 2007; Kikuchi et al. 2011). *Riptortus pedestris* is an economically important polyphagous pest, especially on leguminous plants in East Asia (Bae et al., 2014; Li et al., 2019). For every generation, *R. pedestris* acquires *Burkholderia* spp., symbiotic bacteria belonging to class Betaproteobacteria, from soil environments (Kikuchi et al., 2007, Takeshita and Kikuchi, 2017). The genus *Burkholderia* is comprised of more than

* Corresponding author.

E-mail address: dl343@gachon.ac.kr (D.-H. Lee).

100 species phylogenetically classified into three main clades: plant-associated beneficial and environmental (PBE) clade, *Burkholderia cepacia* and complex (BCC) clade, and stinkbug-associated beneficial and environmental (SBE) clade (Kaltenpoth and Flórez, 2020; Suárez-Moreno et al., 2012; Takeshita and Kikuchi, 2020). Although such phylogenetic classification does not precisely characterize ecological traits of *Burkholderia* clades, the BCC clade includes *Burkholderia* spp. known to be pathogenic to humans or mammals (Chewapreecha et al., 2017; Vial et al., 2011), whereas PBE and SBE clades include bacteria known to be beneficially associated with plants (Suárez-Moreno et al., 2012) and hemipteran insects (Takeshita and Kikuchi, 2017), respectively.

Among species in the SBE clade, *Burkholderia insecticola* (syn. *Caberellonia insecticola*) was isolated and identified from *R. pedestris* adults in Japan (Kikuchi et al., 2005; Shibata et al., 2013; Takeshita et al., 2018). Thereafter, this species has been used almost exclusively in studies for symbiotic associations between *R. pedestris* and *Burkholderia* bacteria. In such studies, laboratory inoculation of *B. insecticola* to *R. pedestris* was conducted and its effects were evaluated in several manners. *Riptortus pedestris* develops sac-like organs at their fourth midgut section called M4 region (Kikuchi et al., 2005) and once *B. insecticola* enters the M4 region, the bacteria can successfully colonize in the insect host (Kikuchi et al., 2011; Kikuchi and Fukatsu, 2014). Although symbiotic *Burkholderia* is not essential for the survival or reproduction of *R. pedestris*, it is known to significantly enhance the fitness of *R. pedestris* by increasing its body size (Kikuchi et al., 2007) and fecundity (Kikuchi and Fukatsu, 2014), or even conferring insecticide resistance (Kikuchi et al., 2012; Sato et al., 2021).

Although substantial research progress has been made with *B. insecticola* for understanding the symbiotic relationship, very few studies have been conducted to elucidate ecological associations between *R. pedestris* and other *Burkholderia* spp. and their potential impacts on insect populations (Jung and Lee, 2019). However, we cannot rule out the possibility that *R. pedestris* could be exposed to a variety of *Burkholderia* spp. including PBE and BCC clades in nature and a portion of them could establish in insects for the following reasons. First, extremely diverse microbes typically coexist in soil, including the *Burkholderia* bacteria complex (Coenye and Vandamme, 2003; Tago et al., 2014). In Japan and South Korea, field soil samples were found to contain mixtures of different *Burkholderia* clades even at very small spatial scales (e.g., < 30 ml) (Jung and Lee, 2019; Tago et al., 2014). Second, although *R. pedestris* has a constricted region in the gut to reduce the risk of infection with non-symbionts or pathogens (Jang and Kikuchi, 2020; Ohbayashi et al., 2015), recent studies suggest that bacteria in PBE and BCC clades may successfully colonize in *R. pedestris*. Jung and Lee (2019) reported that *Burkholderia* species belonging to BCC clade was detected from overwintering *R. pedestris* in South Korea. In addition, Itoh et al. (2019) demonstrated that 12 *Burkholderia* species belonging to PBE clade successfully colonized in the M4 region of *R. pedestris* when individually inoculated under laboratory conditions. Therefore, it is important to include diverse *Burkholderia* clades in future study to better understand ecological associations between *R. pedestris* and the genus *Burkholderia* in nature.

Therefore, in this study, we conducted a nation-wide field survey in South Korea and laboratory experiments to characterize *Burkholderia* clade compositions in *R. pedestris* in the context of the *Burkholderia* complex found in soil environments. For this, soil samples were collected to investigate their *Burkholderia* clade compositions. Then, we provided *R. pedestris* with the field-collected soil as a source of *Burkholderia* acquisition under laboratory conditions, and analyzed *Burkholderia* clade compositions in the insect midguts. Finally, to validate results obtained from laboratory experiments, wild *R. pedestris* were collected and their *Burkholderia* clade compositions were analyzed.

Materials and methods

Sampling sites

Riptortus pedestris is known to use soybean as one of their main food resources, and the insects inhabit and move between crop fields and adjacent forest areas (Kim and Lim, 2010; Kim et al., 2014; Park et al., 2016). Sampling sites cultivating soybean plants were selected in each of the following eight different provinces of South Korea based on Korean Statistical Information Service (KOSIS): Gwangju, Inje, Gongju, Goesan, Andong, Geochang, Gochang, and Muan (Fig. 1). In each province, a sampling site was designated to include a combination of soybean field and adjacent forest as one sampling unit in which *R. pedestris* inhabit (Park et al., 2016). The size of sampling area ranged from 3,292 m² to 10,023 m². The mean sampling areas investigated was 6,450 ± 830 m² (mean ± SE).

Soil sampling

From sampling sites, soil samples were collected every two months from April through October in 2019. In each site, five soil samples were collected from each of soybean field and forested area. Sampling points were located at least 5 m apart from each other. For each sample, 30 ml of soil was collected using a 50 ml conical tube (Hyundai micro Co., Ltd., Seoul, South Korea). A total of 320 soil samples (10 samples × 8 sites × 4 months) were collected and stored at 4 °C until samples were subjected to PCR analysis to identify *Burkholderia* clade compositions from soil itself and provide a source of *Burkholderia* acquisition for *R. pedestris*.

Insects

Riptortus pedestris reared in a laboratory colony was used in this study. The laboratory colony was established with wild *R. pedestris* collected from wooded areas at Gachon University, Gyenggi-do, South Korea (37°27'1.60"N 127°7'50.32"E) using aggregation pheromone traps (Green-Agrotech Co. Ltd, Gyeongsangbuk-do, South Korea). The colony was maintained at 25.5 ± 0.1 °C, 38.3 ± 0.1% RH, and 16L:8D. These insects were provided with dried soybean (*Glycine max*) as a food source and distilled water containing 0.05% ascorbic acid. Once nymphs hatched from eggs, ten individuals were transferred into each breeding jar with distilled water containing 0.05% ascorbic acid and reared to the second instar before use in experiments.

Riptortus pedestris reared on field-collected soil

This experiment was conducted to assess *Burkholderia* clade composition acquired by *R. pedestris* from field-collected soil samples. To facilitate colonization of *Burkholderia* in the soil, soil samples (ca. 3.3 g) were incubated on a piece of cotton soaked with distilled water containing 0.05% ascorbic acid over 48 h prior to experiments. Then, ten individuals of early 2nd instar nymphs (<12 h old) were reared on the soil sample in an insect breeding jar under a light regime of 16:8 (L:D) at 25.10 ± 1.61 °C and 40.12 ± 9.83 % RH. These nymphs were reared to late 3rd or early 4th instar with soybeans as a food source. Among these individuals, five nymphs were randomly selected from each breeding jar and dissected to isolate M4 regions of their midguts in phosphate buffer saline (PBS). A total of 1,600 M4 samples (5 nymphs × 10 soil samples × 8 sites × 4 months) were collected and stored individually in 20 µl PBS. These M4 samples were then subjected to PCR analysis to identify *Burkholderia* clade compositions.

Riptortus pedestris collected from wild populations

To validate results obtained from laboratory experiments, *R. pedestris* adults were collected from the same sampling sites from which the soil samples were collected (Fig. 1). Adults were caught by handpicking or using insect nets every two months from April through October in 2019. A total of 289 adults were collected and dissected to isolate their M4

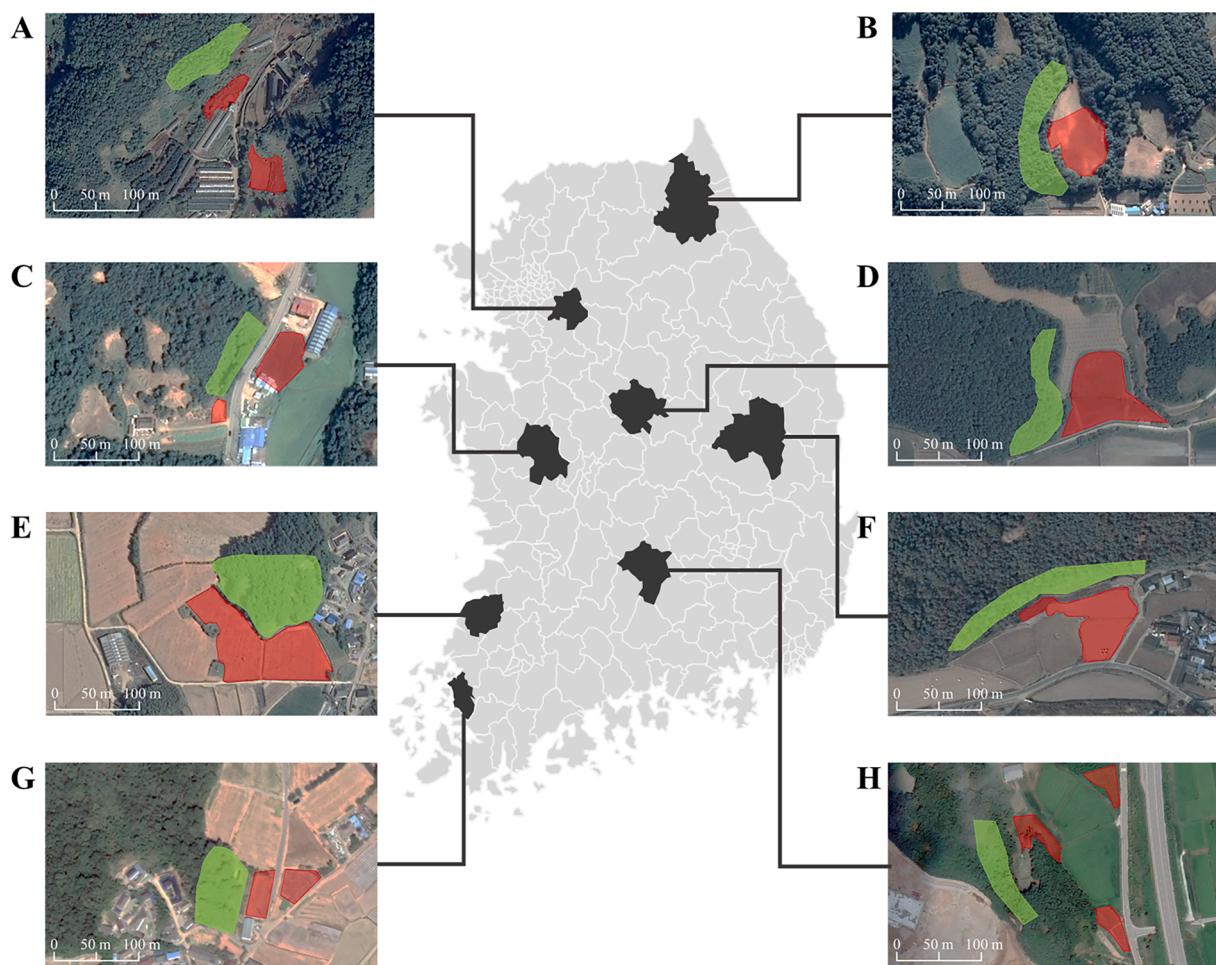


Fig. 1. Sampling sites established in eight provinces of South Korea for collecting soil samples and *Riptortus pedestris*: (A) Gwangju, (B) Inje, (C) Gongju, (D) Goesan, (E) Gochang, (F) Andong, (G) Muan, and (H) Geochang. Green box indicates soybean field. Red box indicates adjacent forested area investigated in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

regions in PBS. Midgut samples were stored individually in 20 µl PBS and subjected to PCR analysis to identify *Burkholderia* clade compositions.

PCR analysis for *Burkholderia* clade identification

Both soil samples and midgut samples were individually subjected to DNA extraction. DNA was extracted for each soil sample (250 mg of soil per sample) using a DNeasy PowerSoil Pro kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. For DNA extraction from midgut sample, a MagListoTM 5 M Genomic DNA Extraction Kit (Bioneer Co. Ltd., Daejeon, South Korea) was used according to the

manufacturer's instructions.

Diagnostic PCR was conducted using *Burkholderia*-specific 16 s rRNA primer sets (Burk16SF and Burk16SR) (Kikuchi et al., 2005) and *Burkholderia*-clade specific primer sets for SBE (SBE160F and SBE1400R) (Itoh et al., 2014), PBE (Burk16SF and PBE822R) (Itoh et al., 2014), and BCC clades (BCC370F and Burk16SR) (Itoh et al., 2014), respectively (Table 1). The temperature profile for diagnostic PCR was as follows: 95 °C for 10 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min (Itoh et al., 2014). In PCR analysis, *Burkholderia* strain RPE75 was included as a positive control to confirm the validity of our method. Samples that were tested positive for genus *Burkholderia* not

Table 1

List of primers used in this study.

Target group	Target gene	Primer name	Direction	Nucleotide sequence (5'→3')	Approximate product size (kb)	Reference
<i>Burkholderia</i>						
<i>Burkholderia</i> spp.	16 s rRNA	Burk16SF	Forward	TTTTGGACAATGGGGCAAC	0.7	Kikuchi et al. 2005
		Burk16SR	Reverse	GCTCTTGGTAGCAACTAAG		
SBE	16 s rRNA	SBE160F	Forward	CGCATACGACTTAAGGGA	1.3	Itoh et al. 2014
		SBE1400R	Reverse	CTTGCCTTAAAGCTACCT		
PBE	16 s rRNA	Burk16SF	Forward	TTTTGGACAATGGGGCAAC	0.5	Itoh et al. 2014
		PBE822R	Reverse	CTTCGTTACCAAGTCAATGAAGA		
BCC	16 s rRNA	BCC370F	Forward	TTTTGGACAATGGCGAAAG	0.8	Itoh et al. 2014
		Burk16SR	Reverse	GCTCTTGGTAGCAACTAAG		
Bacteria	16 s rRNA	16SA1	Forward	AGAGTTGATCMGGCTCAG	1.5	Fukatsu and Nikoh, 1998
		16SB1	Reverse	TACGGYTACCTTGTACGACTT		
Invertebrates	COI	LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	0.7	Folmer et al., 1994
		HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA		

belonging to SBE, PBE, or BCC clade were categorized as ‘unidentified’ clade.

In addition, to validate DNA extraction was appropriately performed, additional diagnostic PCR was conducted when sample was found *Burkholderia*-negative. Eubacterial 16 s rRNA primer sets (16SA1 and 16SB1) were used for soil samples (Fukatsu and Nikoh, 1998); invertebrates’ mitochondrial cytochrome oxidase I (COI) gene specific primer sets (LCO1490 and HCO2198) were used for insect samples (Forlmer et al., 1994) (Table 1). Diagnostic PCR for targeting eubacterial 16 s rRNA in soil was performed at 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 70 °C for 2 min (Fukatsu and Nikoh, 1998). Diagnostic PCR for targeting insect COI gene in *R. pedestris* was performed at 95°C for 1 min, followed by 30 cycles of 95°C for 1 min, 48 °C for 1 min, and 72 °C for 1 min (Fukatsu and Nikoh, 1998). From this additional PCR analysis, it was confirmed that DNA was successfully extracted from all the *Burkholderia*-negative samples.

Statistics analysis

Detection rates of genus *Burkholderia* from soil samples and *R. pedestris* reared on the field-collected soil were compared respectively across provinces and months using analysis of variance (ANOVA) (JMP, Version 12). For *R. pedestris* adults collected from field, detection rates were compared between females and males using *t*-test (JMP, Version 12).

Results

Burkholderia in field-collected soil

From the field-collected soil samples, 88.44% were found to contain genus *Burkholderia*. Mean detection rates of genus *Burkholderia* in eight sampling sites varied from 75.00% to 100.00% ($F = 3.6620$, $df = 7, 24$, $P = 0.0079$) (Fig. 2A). The detection rates of genus *Burkholderia* in Andong

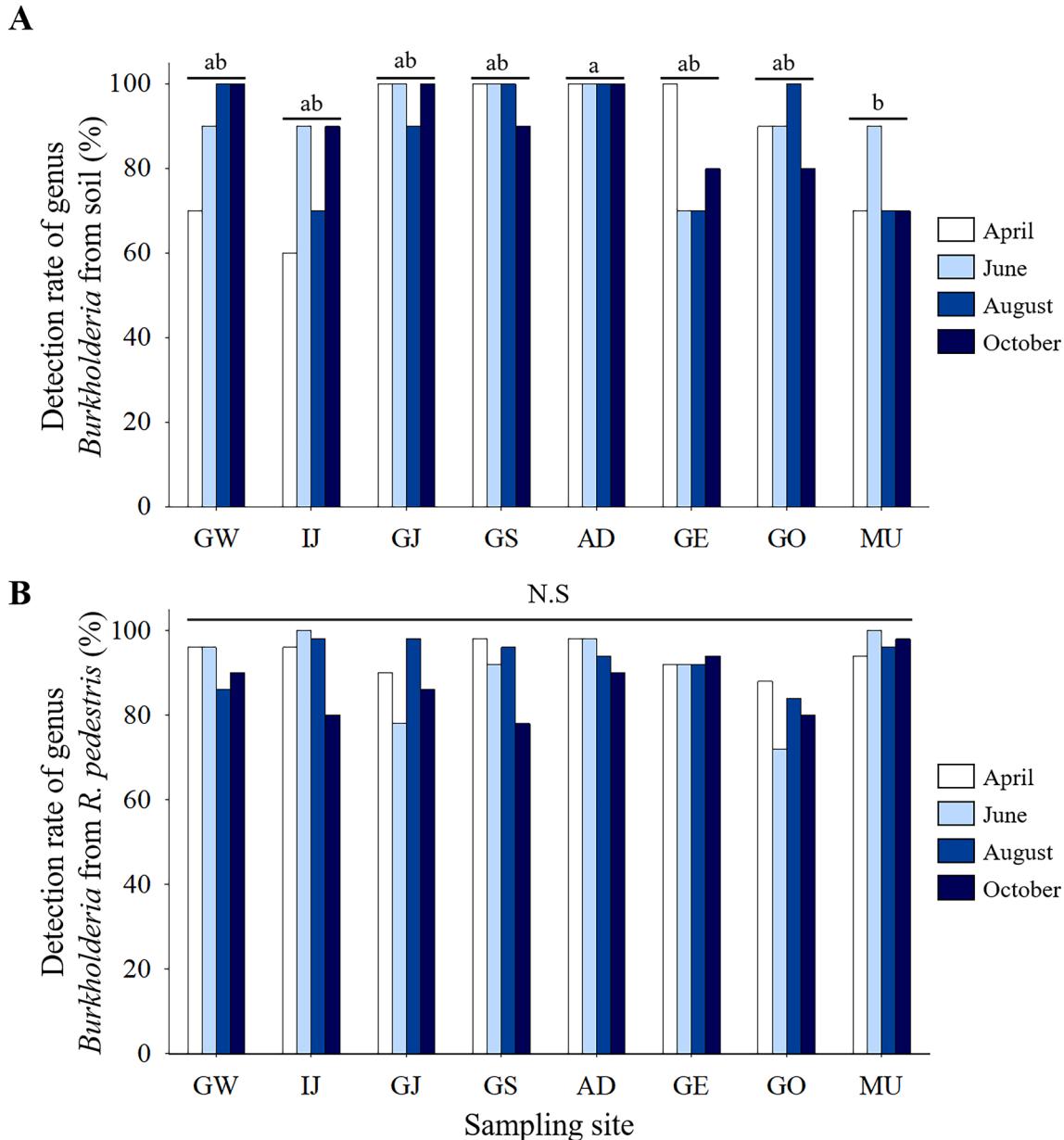


Fig. 2. Detection rates of genus *Burkholderia* from (A) field-collected soil samples and (B) *Riptortus pedestris* reared on soil samples. Different letters indicate significant difference in detection rate between sampling sites ($p < 0.05$; ANOVA (analysis of variance) followed by Tukey’s HSD (honestly significant difference)). Abbreviation: GW, Gwangju; IJ, Inje; GJ, Gongju; GS, Goesan; AD, Andong; GE, Geochang; GO, Gochang; MU, Muan.

was the highest, whereas that in Muan was the lowest. Over the sampling period, mean detection rates of genus *Burkholderia* in April, June, August, and October were 86.25%, 91.25%, 87.50%, and 88.75%, respectively. These rates were not significantly different among sampling months ($F = 0.1997$, $df = 3,28$, $P = 0.8957$) (Fig. 2A).

The detection rate of triple clade was the highest, with 42.81% of soil

samples containing all three identified clades consisting of SBE, PBE, and BCC clades (Fig. 3A). Detection rates for double and single clades were 26.25% and 19.06%, respectively. In addition, only one sample was detected with an unidentified *Burkholderia* clade, which did not belong to any of the three identified clades. These results were also analyzed to evaluate how frequently each of the three clades was

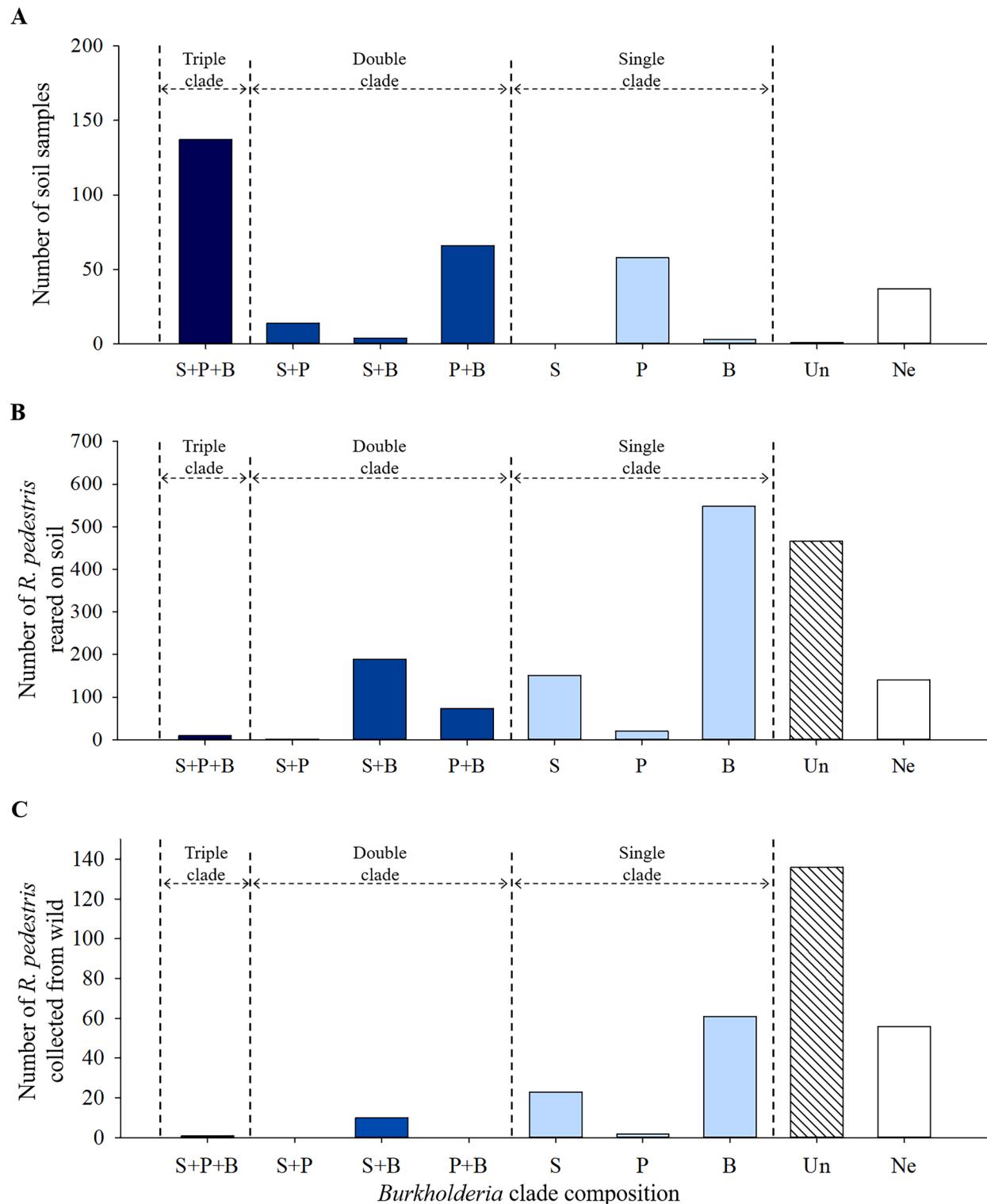


Fig. 3. Numbers of (A) field-collected soil samples, (B) *Riptortus pedestris* reared on soil samples, and (C) *R. pedestris* collected from wild populations detected with different *Burkholderia* clade compositions. Abbreviation: S, SBE clade; P, PBE clade; B, BCC clade; Un, Unidentified clade; Ne, Negative. Negative indicates the case from which *Burkholderia* bacteria are not detected.

detected from soil and insect (Fig. 3). For example, if a soil sample was detected with both SBE and PBE, then the detection frequency in Fig. 3 was added by one for both SBE and PBE. Results showed that PBE clade (42.97%) was the most frequently detected, followed by BCC (32.81%) and SBE (24.22%) clades (Fig. 4A).

Burkholderia in *R. pedestris* reared on field-collected soil

When *R. pedestris* were reared on field-collected soil, 91.25% of these insects were found to harbor genus *Burkholderia*. Mean detection rates of genus *Burkholderia* varied from 81.00% to 97.00% among the eight sampling sites. However, there was no significant difference in the detection rate among sampling sites ($F = 2.3742$, $df = 7, 24$, $P = 0.0539$) (Fig. 2B). Over the sampling period, 94.00%, 91.00%, 93.00%, and 87.00% of *R. pedestris* harbored the genus *Burkholderia* when they were reared on soil samples collected in April, June, August, and October, respectively (Fig. 2B). There was no significant difference in the detection rate among sampling months ($F = 1.4991$, $df = 3, 28$, $P = 0.2364$).

Among the three identified clades, the detection of single clade was the most dominant from *R. pedestris* reared on soil samples, with 44.94% of insects harboring single clade, followed by double (16.56%) and triple (0.63%) clades (Fig. 3B). In addition, 29.13% of insects were found to harbor the unidentified *Burkholderia* clade. Among identified *Burkholderia* clades, BCC clade (64.19%) was the most frequently detected, followed by SBE (27.52%) and PBE (8.29%) clades (Fig. 4B).

Burkholderia in *R. pedestris* collected from field

Over the sampling period, a total of 289 *R. pedestris* adults (Female: Male = 146:143) were caught from sampling sites. In general, for both females and males, the number of adults rapidly increased between August and October (Fig. 5). The mean detection rates of genus *Burkholderia* were 90.06% and 84.95% for females and males, respectively, over the study period: there was no significant difference between females and males ($t = 1.03$, $df = 45$, $P = 0.3107$). The detection rates varied from 72.73% to 100.00% in females and from 64.47% to 100.00% in males (Fig. 5). Among the three identified *Burkholderia* clades, single clade was detected the most dominant from wild *R. pedestris*, 29.76% of insects collected (Fig. 3C). Detection rates of double and triple clades were 3.46% and 0.35%, respectively. In addition, 47.06% of *R. pedestris* were found to harbor the unidentified clade. Among the three identified *Burkholderia* clades, BCC clade (66.06%) was the most frequently detected, followed by SBE (31.19%) and PBE (2.75%) clades (Fig. 4C).

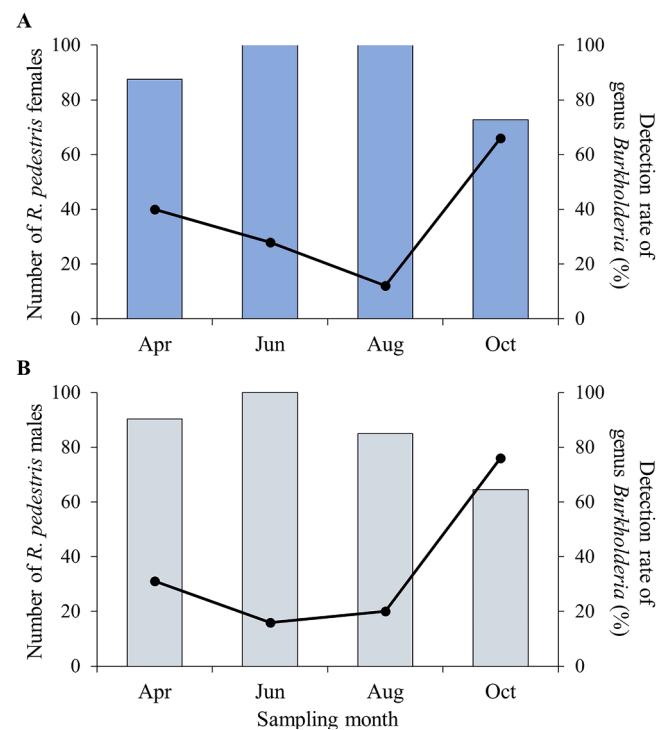


Fig. 5. Numbers of *Riptortus pedestris* (A) females and (B) males collected from sampling sites and their detection rates with genus *Burkholderia*. Line indicates the number of *R. pedestris*. Vertical bar indicates the detection rate.

Discussion

Results of this study indicate that the genus *Burkholderia* are prevalent and widely distributed in soybean fields and adjacent forests across South Korea. Soil sampling and subsequent PCR analysis revealed that ca. 88% of soil samples contained single or multiple *Burkholderia* clades. In addition, our results demonstrate that *R. pedestris* nymphs can readily acquire *Burkholderia* spp. from field-collected soil, resulting in ca. 91% of the reared insects to successfully harbor these bacteria in their midguts. A similar pattern was observed in Japan. When *R. pedestris* individuals were reared on field-collected soil during the 2nd instar period and ca. 76% of those individuals acquired genus *Burkholderia* (Kikuchi et al., 2011). Indeed, field sampling of *R. pedestris* in this study confirms that *R. pedestris* could acquire genus *Burkholderia* with high likelihood in nature: ca. 81% of wild individuals collected from soybean fields across South Korea were found to harbor genus *Burkholderia*.

This study characterized *Burkholderia* clade compositions in both

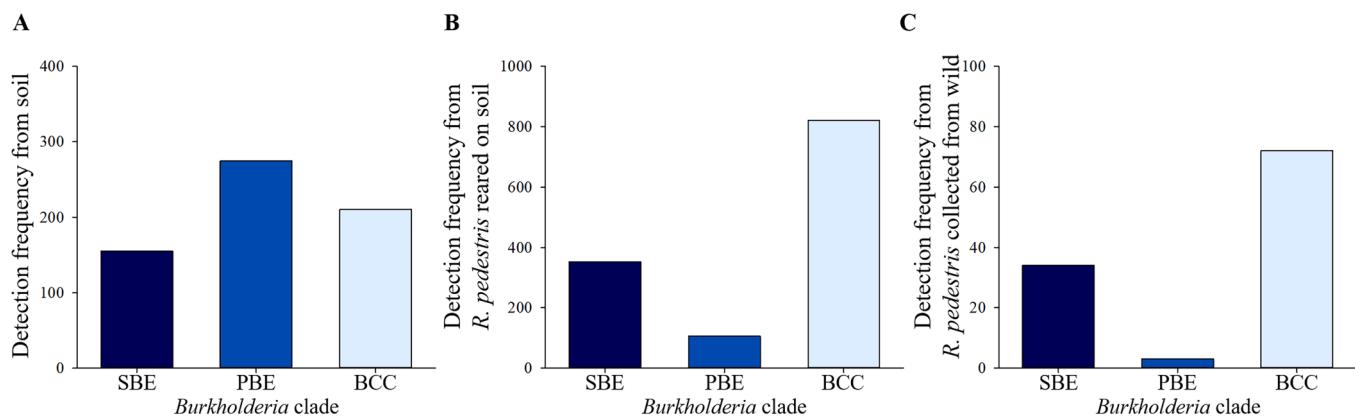


Fig. 4. Detection frequencies of three identified *Burkholderia* clades from (A) field-collected soil samples, (B) *Riptortus pedestris* reared on soil samples, and (C) *R. pedestris* collected from wild populations. Detection frequency is the number of times being detected with each of the three clades from soil or insect (see Results).

field soil samples and *R. pedestris* reared on those soil samples for the first time, thereby allowing us to better understand relationships of clade compositions between soil and insects. From soil analysis, ca. 69% of samples were found to contain multiple clades consisting of the three identified *Burkholderia* clades (SBE, PBE, or BCC). When *R. pedestris* were reared on field soil, all three identified clades successfully colonized the insect. That is, *Burkholderia* spp. in these clades can go through constricted region (CR), a filter-like organ, in the gut track of *R. pedestris* and subsequently colonize the M4 midgut region (Itoh et al., 2019; Jang and Kikuchi, 2020; Jung and Lee, 2019; Ohbayashi et al., 2015). Interestingly, the prevalence pattern of *Burkholderia* clade compositions in *R. pedestris* was completely reversed compared to that observed from soil samples: the detection of single clade was dominant, whereas triple clade was detected from < 1% of *R. pedestris* evaluated. Taken together, *R. pedestris* is likely to contact and intake multiple *Burkholderia* clades while foraging in soil environments, although the establishment of a single clade could follow due to differential survival among *Burkholderia* clades in the midgut of *R. pedestris*.

Under laboratory conditions, Itoh et al. (2019) found that a SBE clade (*B. insecticola*) outcompetes a PBE clade (*B. fungorum*) in the midgut of *R. pedestris*. Indeed, when *R. pedestris* were reared on soil samples containing all SBE, PBE, and BCC clades in this study, the SBE clade was detected 6.06 times greater than the PBE clade. Also, from wild *R. pedestris* populations, the detection frequency of SBE clade was 11.33 times greater than that of PBE clade. Moreover, very few *R. pedestris* were found to harbor SBE and PBE clades together when reared on the field-collected soil. For wild *R. pedestris*, none was found to harbor SBE and PBE clades together. Therefore, given that PBE clade was the most dominant clade in field soil, competitive exclusion of PBE clade by SBE in *R. pedestris* might have played a role in shaping the observed clade composition patterns from insects.

Contrast to results for SBE and PBE clades in *R. pedestris*, high levels of BCC clade detections in both laboratory-reared and wild *R. pedestris* are somewhat unexpected. Itoh et al. (2019) demonstrated in laboratory experiments that *Burkholderia* sp. belonging to BCC clade did not stably colonize the M4 midgut region. However, for both laboratory-reared and wild *R. pedestris* in this study, the detection frequency of BCC clade was the highest among the three identified clades. BCC clade was also found the most frequently from *R. pedestris* as a single clade without coexistence with the other two identified clades. One possibility for this discrepancy between the two studies may root from genetic variation of *R. pedestris* between South Korea and Japan. For example, a previous study found that pea aphids have extensive genetic variation in the ability to regulate their heritable bacterial symbiont *Buchnera aphidicola* (Chong and Moran, 2016). Further studies are warranted to characterize genetic variation of *R. pedestris* across large geographical scales and address how the variation would affect symbiosis with the genus *Burkholderia*.

Our results indicate that *Burkholderia* spp. designated to an unidentified clade in this study could successfully colonize in *R. pedestris* to the extent comparable or exceeding the identified clades such as BCC. Likewise, a recent field survey in South Korea reported that ca. 70% of *R. pedestris* adults were detected with *Burkholderia* spp. not belonging to any of SBE, PBE, or BCC (Jung and Lee, 2019), which was higher than the proportion of the unidentified clade found in this study. This difference may result from dissimilarity of either geographical location or season of field sampling of *R. pedestris*. These unidentified *Burkholderia* spp. should be identified at species level to define their phylogenetic status and thereby better understand evolutionary relationships between genus *Burkholderia* and *R. pedestris*. Also, ecological effects of those bacteria on insect host need to be addressed in a future study.

Finally, when *R. pedestris* nymphs were reared on the field-collected soil, ca. 32% of these insects were found to harbor *Burkholderia* clades which were not detected from soil analysis (see Supplementary Fig. S1). This discrepancy, in part, may be the result of ecological traits of organisms in this study. Soil microbes are extremely heterogeneous in

abundance and distribution even within a few centimeters (Morris, 1999; O'Brien et al., 2016). Given that only a small fraction of soil sample (e.g., 250 mg) is typically used for the analysis (Bach et al., 2018; Penton et al., 2016), the observed discrepancy between soil and insect within replicate is thought to be the norm rather than exception. For this reason, we analyzed a large number of replications, consisting of 320 soil samples and 1,600 insects, and documented the general patterns of *Burkholderia* clade compositions in soil and insect.

In summary, results of this study provide baseline information to better understand ecological associations between *R. pedestris* and *Burkholderia* spp. in different clades. First, our field survey confirms that genus *Burkholderia* are prevalent and widely distributed in South Korea. Second, *R. pedestris* would be exposed to diverse *Burkholderia* spp. in soil environments; however, these insects are more likely to be colonized by a single *Burkholderia* clade such as BCC, possibly resulting from competition among different clades. Further laboratory experiments are warranted to investigate competitions between different *Burkholderia* clades in *R. pedestris* present in South Korea. Third, our data strongly suggest that, in addition to SBE clade, potential effects of other *Burkholderia* clades on the fitness or behavior of *R. pedestris* need to be elucidated. Finally, *Burkholderia* spp. in the unidentified clade should be identified and also studied for their associations with *R. pedestris*.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aspen.2022.101976>.

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