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Discovery and Biology of *Spathius verustus* Chao (Hymenoptera: Braconidae), a Potential Biological Agent on Two *Monochamus* Vectors of the Pinewood Nematode

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Abstract: Pine wilt disease in Korea can be managed by reducing vector density through chemical application. To reduce the side effects from pesticides, effective natural enemies must be identified and used to reduce the vectors' natural density. Sentinel logs were used to investigate a parasitoid wasp parasitic to *Monochamus alternatus* and *Monochamus saltuarius*, the vectors of *Bursaphelenchus xylophilus*, which causes this disease. During 2016–2017, the parasitoid wasps distributed in the *Pinus densiflora* and *Pinus koraiensis* forests were surveyed using sentinel logs at six different areas. An ectoparasitoid wasp *Spathius verustus* Chao of *M. alternatus* and *M. saltuarius* was identified. We showed for the first time that *S. verustus* was parasitic to *M. alternatus* in South Korea and to *M. saltuarius* worldwide. The parasitism rates were affected by region and session but not by the sentinel log height in the tree and the distances between the trap and forest edge locations. Studies also showed that *S. verustus* appeared to prefer *M. alternatus* to *M. saltuarius* as a host. This study unveiled the ecological details of *S. verustus*. Further research on various environmental factors such as regional differences, host density differences, and the degree of damage from the pine wilt disease is required to understand the effects of environmental or ecological factors on parasitism rates.

Keywords: biological control agent; ectoparasitoid; Braconidae; *Monochamus* vector; pine wilt disease; parasitism rate

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1. Introduction

Pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer), native to North America, causes pine wilt disease (PWD) in East Asia (Japan, China, Taiwan, and South Korea), Mexico, and Europe (Portugal and Spain), inflicting serious damage on pine forests around the world [1–8]. The first damage on pines in Korea was reported by Yi et al. [9]. The pinewood nematode is known to infest various species of *Pinus*, *Abies*, *Chamaecyparis*, *Cedrus*, *Larix*, *Picea*, and *Pseudotsuga*, all of which belonged to Pinales [10]. The hosts of the pinewood nematode in Korea include *P. densiflora* (Siebold & Zucc), *P. koraiensis* (Siebold & Zucc), and *P. thunbergii* (Parl), as well as *P. parviflora* Siebold & Zucc in some insular areas [11]. The pinewood nematode spreads among its hosts by using wood-boring beetles such as long-horned beetles from the genus *Monochamus* as vectors [12–14].

The representative insect vectors of PWN are, in particular, the species of *Monochamus* in Cerambycidae, and there are eight species known to the Korean insect fauna [15]. Of them, two native species, that have never had a relationship with PWN, have come to transmit PWN in the country since the invasion: *M. saltuarius* Gebler as the vector of PWN in the central region and *M. alternatus* Hope in the southern region of the country [16]. The pinewood nematode, transmitted by *M. alternatus*, has been causing immense damage to coniferous forests in Jeju Island since 2013 [17]. PWD causes a higher mortality rate than the pine caterpillar (*Dendrolimus spectabilis* Butler) or pine needle gall midge (*Thecodiplosis japonensis* Uchida & Inouye), causing great economic damage to the Korean forestry industry. The area affected by PWD has increased from 72 ha in 1988 to 6325 ha in 2016 [18].

Currently, the methods used in Korea to manage PWD include spraying insecticide using unmanned aerial vehicles, fumigation or incineration after harvesting damaged trees, and clear-felling around damaged trees [19,20]. However, these control methods negatively affect the environment and require a large budget and human capital for the processes of scientific forecasting with manned and unmanned aerial vehicles, as well as restoring forests [21,22]. Additionally, Jeon et al. [23] argued that clear-felling would further spread PWD. One of the ways to overcome these drawbacks to certain degrees is biological control using natural enemies to reduce the density of the insect vectors.

There have been many attempts to use natural enemies, such as fungus, parasitoids, or predators, to control populations of cerambycid vectors. For example, *M. alternatus* also transmits the pinewood nematode in China [24], where, to prevent the disease, *Sclerodermus harmandi* (Buysson) (Hymenoptera: Bethylidae) was used as a biological control agent (parasitism rate from 42.8 to 46.1%; average control effectiveness of 65.3%) [25]. There also has been a study on the indoor mass-rearing of *S. harmandi* using *Tenebrio molitor* Linnaeus as an alternative host [26]. As in South Korea, *M. alternatus* and *M. saltuarius* also transmit the pinewood nematode in Japan [27,28]. Several studies have addressed biological control methods such as using an entomopathogenic fungus, particularly *Beauveria bassiana* (Bals.—Criv.) Vuill., hymenopteran parasitoids, and coleopteran predators [29,30].

In Korea, research on the biological control of PWD has been conducted since 2004 [31] and highlighted the biological characteristics of *S. harmandi* as a natural enemy of *M. alternatus* and *M. saltuarius*. In indoor tests using small glass vials in a controlled laboratory environment, the parasitism on *M. saltuarius* larvae was as high as 98.6%, and it was selected for mass-rearing to reduce the density of *M. alternatus* and *M. saltuarius* [32]. However, there has been no report on the effectiveness of the bethylid wasp from outdoor release, if any, so far [33]. The reason for not having any report on actual usage of the bethylid wasps seems to be related to the host range. According to Lim et al. [34], the hosts of *S. harmandi* include more than 30 species of 9 insect families, which means the parasitoid is not a specialist parasitoid to the targeted cerambycids but a generalist. Further, Liu et al. [35] demonstrated the adverse effects on an important natural enemy (*Triaspis* sp. of Braconidae) of the pine weevil in China and suggested reconsideration of further release of *S. harmandi* for biological control programs. Since the first biological control attempt using *S. harmandi*, there has been little research on natural enemies or developing biological control methods on two *Monochamus* vectors in South Korea. Kim et al. (2010) [36] conducted an extensive study to find the natural enemies of various wood-boring insect pests, and of the surveyed parasitic wasps, two ichneumonid species were reared from prepupae and pupae of *M. saltuarius*. In Portugal, a relatively high diversity of parasitoids was also found associated with *M. galloprovincialis*, the pinewood nematode insect vector, but they are mainly idiobiont ectoparasitoids and generalists that attack a vast array of other insects living in dead and dying trees [37].

Great examples of the most active research on biological control development against wood-boring pests can be found in the U.S. and Europe, in which we can follow their natural enemy survey methods or strategies to develop a biological control program on the insect vectors, which are also wood-boring insects, of PWN. Three coleopteran invasive species inflict tremendous damage on hardwoods in those regions: *Anoplophora chinensis* (Förster) and *A. glabripennis* (Motschulsky) in Cerambycidae, and *Agrilus planipennis* Fairmaire in Buprestidae. Surveys for natural enemies, particularly parasitoids, have been conducted for over ten years to be applied to biological control programs (e.g., [38–41]). Especially, in the U.S., various studies have been carried out using sentinel logs to find natural enemies such as egg, larval, or pupal parasitoids that are suitable for biological control against the three wood-boring insect pests [42–45]. In particular, a biological control plan to stem the infestation of the emerald ash borer (*A. planipennis*) was initiated back in 2003, and groups of scientists from the U.S., China, and Russia searched for effective biological control agents in East Asian regions, from which the invasive pest was originated. Four parasitoids were proven to be effective and released to the fields for biological control since 2007. These parasitoids are *Oobius agrili* Zhang and Huang, an egg parasitoid in Encyrtidae; *Spathius agrili* Yang and S. *galinae* Belokobylskij & Strazanac, two larval parasitoids in Braconidae, and *Tetrastichus planipennisi* Yang, a larval parasitoid in Eulophidae. Several millions of them have been released, and the parasitoid species are found to be established in many states of the U.S. up to date (e.g., [46–49]).

It is imperative to find effective parasitoids that can be used with other control methods such as removing infested trees and injecting nematicides into trunks. Additionally, compared to other control methods, biological control can be cost-effective in the long term after parasitoids are successfully established in released areas. As the first step for biological control development, the present study aimed (1) to find potential parasitoids, particularly on eggs or larvae, of the two *Monochamus* insect vectors of the pinewood nematode using sentinel logs, and (2) to investigate ecological and biological characteristics of parasitoids in prior to the development of a biological control program and strategies.

2. Materials and Methods

2.1. Sentinel Log Preparation

To search for natural enemies of *M. alternatus* and *M. saltuarius*, we adopted the host egg-sentinel log method used by Duan et al. [50]. The sentinel logs used in our study are Korean pine (*P. koraiensis*) logs artificially infested with the two *Monochamus* species [51]. The reasons for selecting the Korean pine for the sentinel logs were that the larvae of *M. saltuarius* were known to have high survival in the Korean pine, and that *M. alternatus* preferred it over the Korean red pine (*P. densiflora*) for oviposition [52,53]. Preparation of the sentinel logs was carried out by (1) cutting freshly chopped Korean pine wood into split logs with bark intact (width 15 × length 25 × thickness 5 cm). The sentinel logs of this size were found to be advantageous for the field survey in terms of transportation and installation. (2) Two Korean pine blocks and ten pairs of adult vectors were placed in a plastic box (50 × 40 × 20 cm) and induced to lay eggs for three to four days. This was performed separately for each *Monochamus* species, avoiding them infesting the same split logs. (3) A hole was drilled near the top of a sentinel log for hanging. It is crucial that the sentinel logs should be installed in the field right after preparation to catch egg or larval parasitoids because the egg stage of *M. alternatus* and *M. saltuarius* only lasts about five to ten days according to Ochi [54].

2.2. Survey Site

Survey areas were chosen according to three criteria: (1) affected by PWD; (2) no aerial pesticide application; and (3) recorded presence of the insect vectors. The information of PWN-infested areas was provided by the National Institute of Forest Science, South Korea. *M. alternatus* is mainly distributed in the *Pinus densiflora* forests from the central to the southern region, including Jeju Island; *M. saltuarius* is mainly distributed in the *P. densiflora*, the Korean red pine, and *P. koraiensis*, the Korean pine, in forests from the central to the northern region [16]. Based on this information, the search for parasitoids of *M. alternatus* was first conducted in three Korean red pine stands of three southern locations in 2016: Jinju (N35.130196 E128.100493) and Geoje (N34.563059 E128.402835) in Gyeongsangnam-do, and Suncheon (N34.582965 E127.283301) in Jeollanam-do. In 2017, the parasitoid survey of *M. saltuarius* was conducted based on a more detailed survey plan in three Korean pine stands in three northern locations: Hongcheon (N37.454955 E127.533691) in Gangwon-do, and Yangpyeong (N37.372783 E127.390890) and Gapyeong (N37.511048 E127.285610) in Gyeonggi-do (Figure 1).

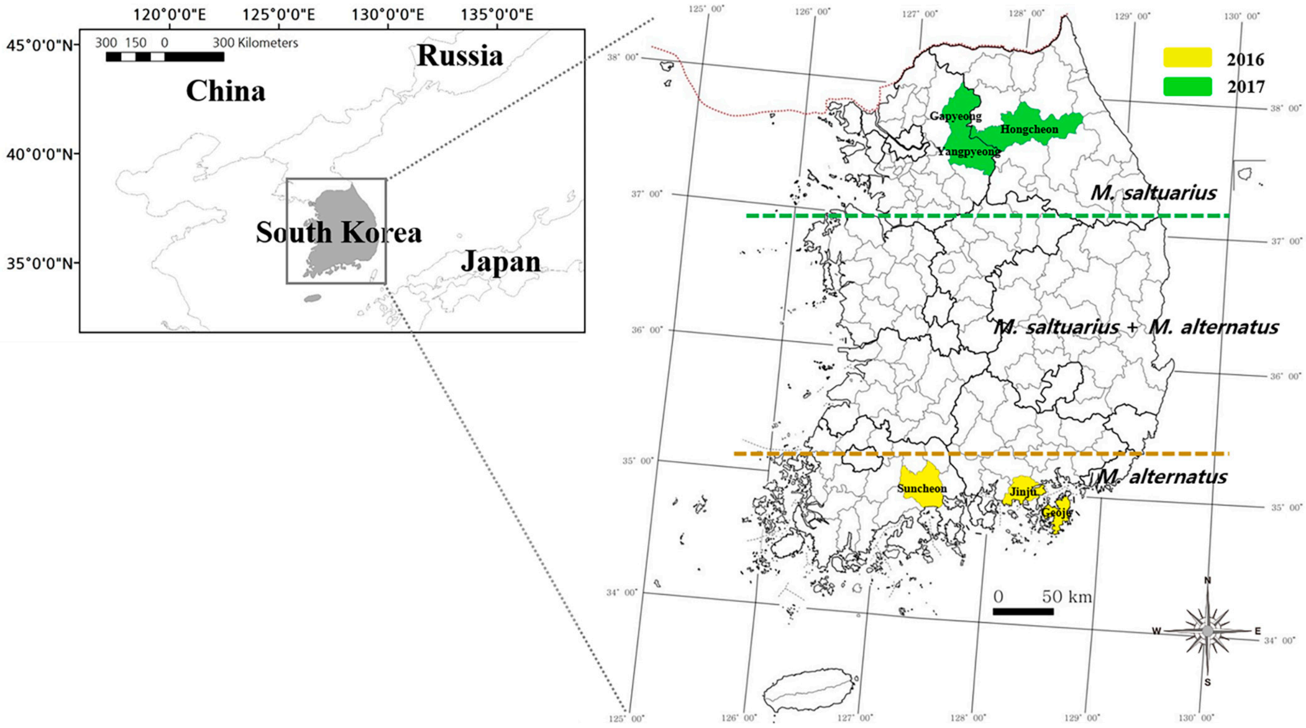

Figure 1. Parasitoid survey sites in South Korea in 2016 (yellow; Suncheon, Jinju, and Geoje) and 2017 (green; Gapyeong, Yangpyeong, and Hongcheon).

2.3. Field Survey

The first field survey was carried out as a pilot test in August 2016. The surveys were conducted twice, each in 14 days, at three sites in the southern region of South Korea (Figure 1). Each survey was conducted with three repeats of the *M. alternatus* sentinel log installations at each site. Along the edge of a Korean red pine stand, two sentinel logs (i.e., one sentinel log installation) were attached to the trunk of a pine tree at each of two different heights (0 and 1.8 m above the ground), adopting the method proposed by Duan et al. (Figure 2A) [48]. In 2017, the survey plan was revised to include more repeats during a longer period than the previous year. The surveys were made four times from mid-May to late July, which is the flight period for *M. saltuarius* according to Han et al. [55], at three sites in the northern region of South Korea (Figure 1). This was designed to investigate the differences in parasitoids composition in the southern and northern parts of Korea. In both 2016 and 2017, the sentinel logs installed were replaced every 14 days after the initiation until the end of the surveys, and after the 14-day exposure, the sentinel logs were brought back to the Korea National Arboretum lab for dismantling (Figure 2B). The barks of the collected sentinel logs were carefully peeled off using a small knife to find host eggs or larvae that were parasitized while recording all the number of host eggs and larvae that were found under the barks to calculate the parasitism rate.

Figure 2. Sampling parasitoids of a vector with a sentinel log of *Monochamus* sp. and indoor oviposition observation setup for *Spathius verustus*: (**A**) hanging two sentinel logs at two different heights (0 m and 1.8 m) from the base of a pine tree; (**B**) peeling off the bark of the collected sentinel logs after 14-day exposure to find parasitoid larvae. (**C**) A whole sentinel log with *S. verustus* in a glass cage. (**D**) Four females (black arrows) gathered in one place, drilling for oviposition.

2.4. Rearing and Identification of the Parasitoid

Collected larvae of the parasitic wasps were reared in small plastic holders (2 × 2 × 2 cm) under room temperature conditions (25 ± 1 °C) until adults emerged. The emerged parasitoids were killed with alcohol, dried using hexamethyldisilazane (HMDS), and card-mounted. Photographs were taken with a Leica DFC 495 camera through a Leica M205A Stereozoom stereomicroscope (Leica, Microsystems, Solms, Germany), and LAS software (version 4.1.0., Leica Microsystems, Switzerland) was used for image stacking. The images were adjusted using Adobe Photoshop CS6 (Adobe Systems Incorporated, San Jose, CA, USA). The parasitic wasp was identified according to the identification key by Tang et al. [56].

2.5. Indoor Oviposition Observation for the Parasitoid

For indoor mass-rearing of the parasitoid wasp in the future, it is crucial to study the host-seeking and oviposition behavior of the parasitoid, which is to find semiochemical or physical cues for oviposition. A simple oviposition test was performed with female wasps in the laboratory. First, early instar larvae of the vectors (= host larvae) were prepared under three different conditions to see to which condition the parasitoid reacts: (1) host larvae completely exposed; (2) larvae placed in a sandwich made of two *P. koraiensis* bark pieces (4 × 4 cm) with oviposition scars made by the vectors; and (3) larvae placed in a sandwich made of two *P. koraiensis* bark pieces (4 × 4 cm) without oviposition scars of the vectors. Each of the larvae was exposed to a pair of the parasitoids, which were fed with diluted honey (5%) for seven days after emergence, in a Petri dish (ø10 cm) to observe the oviposition behavior. Oviposition scars have traces of secretions during the oviposition of the vectors, and these substances (such as long-chain hydrocarbons, ketones of fatty acids, esterified cholesterols, and proteins) act as kairomones to attract parasitoids [57]. We also set up another test in which a dozen female wasps were exposed to a whole sentinel log (width 15 × length 25 × thickness 5 cm) where young host larvae were growing and supposedly feeding actively under the bark (Figure 2C). According to Wang et al. [58], *Spathius agrili* Yang, which is a braconid parasitoid of the emerald ash borer, responded to vibration signals from host-feeding and movement.

2.6. Data Analysis

Correlation analysis, *t*-test, and ANOVA were performed using the rstatix package [59] in R [60] to compare the relationships between variables as follows: (1) size of parasitized host larvae × number of *S. verustus* (the head capsule size of parasitized host larvae was measured, and the number of parasitoid progeny on a host was counted); (2) size of parasitized host larvae × size of female *S. verustus* (the head capsule size of parasitized host larvae was measured, and the hind tibia length of female parasitoid progeny on a host was measured); (3) host density × sex ratio (the number of hosts in a sentinel log and sex ratio of parasitoid progeny on a host were counted); and (4) host density × the number of parasitized host larvae (the number of hosts in a sentinel log and the number of parasitized host larvae were counted).

We also analyzed the effect of environmental variables on the parasitism rate of *S. verustus* with the generalized linear mixed model (GLMM) using the lme4 package [61] in R. The GLMM analysis method was used because the parasitism distribution was suggested to be binomial [62]. In the data of the present study, the fixed effect is the parasitism rate, and the random effects are site, height of sentinel log, forest depth, and session. Wilcoxon test was performed using the stats package [60] to analyze the statistical significance of the relationship between the parasitism rate of *S. verustus* and *Monochamus* species.

2.7. Depository

All materials examined are deposited in the Entomological Collection of the Korea National Arboretum (KNAE), Pocheon, Republic of Korea.

3. Results

3.1. Taxonomic Account of Spathius verustus

Family Braconidae
Genus *Spathius* Nees, 1819

Spathius verustus Chao, 1977

Diagnosis (based on [63,64]). Head behind eyes weakly convex anteriorly and evenly narrowed posteriorly. Transverse diameter of eye 1.1–1.3 times as long as temple. Ocelli arranged in triangle with base 1.2–1.3 times as long as its sides. Palpi light reddish brown or yellow. Mesopleuron always smooth in lower half. Forewing with darkened areas along the veins; vein 2-SR about 1.5 times as long as vein 3-SR. Hind tibia with long pale near the base. Ovipositor slightly shorter than metasoma.

Male (Figure 3A). Body length 2.64 ± 0.29 mm, fore wing length 1.6 ± 0.21 mm ($n = 20$). Antennae with 20–23 flagellar segments. Body saddle brown, legs pale yellow, tarsal claw blackish, metasoma evenly blackish posteriorly.

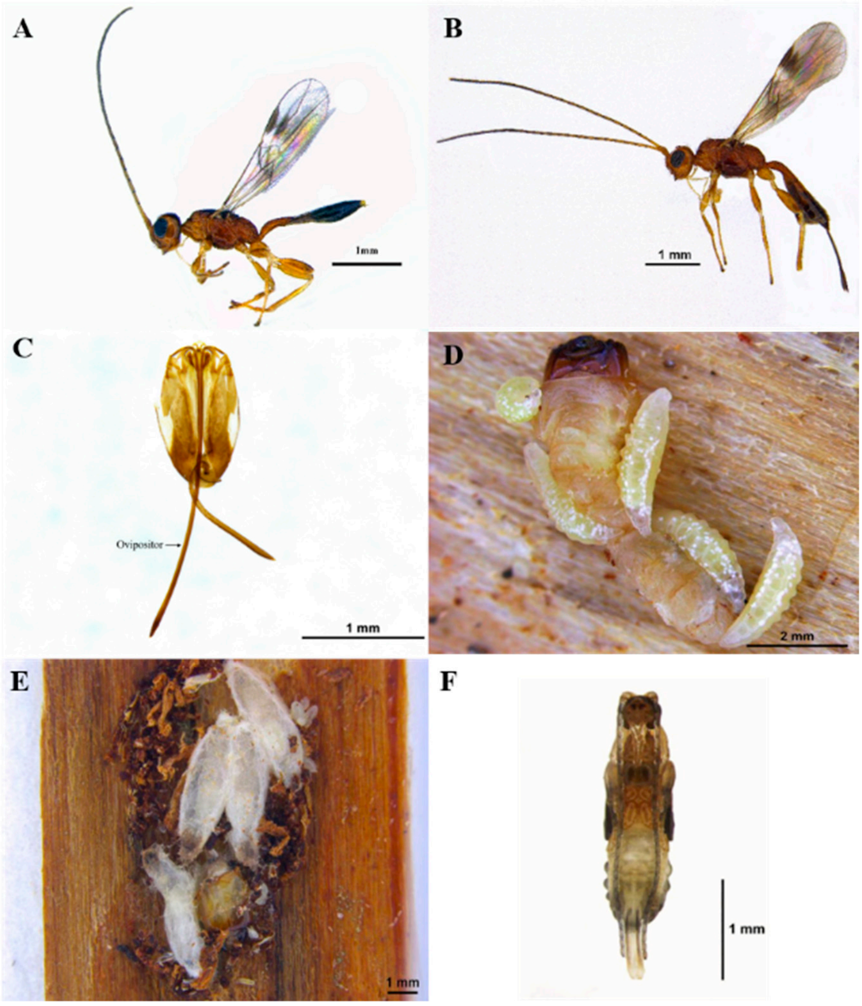

Figure 3. *Spathius verustus*: (**A**) male habitus, lateral view; (**B**) female habitus, lateral view; (**C**) female genitalia; (**D**) mature larvae of *S. verustus* on a larva of *M. alternatus*; (**E**) cocoons near the dead host in the tunnel dug by the host; (**F**) Pupa, female.

Female (Figure 3B, C). Body length 4.21 ± 0.68 mm, fore wing length 2.2 ± 0.27 mm, ovipositor length 1.99 ± 0.25 mm ($n = 20$). Antennae with 22–29 flagellar segments. Body saddle brown, tarsal claw blackish, metasoma evenly blackish posteriorly. Tergite 3 of metasoma to ovipositor black. Mesoscutum, axilla, and scutellum narrow and black. Fore and middle legs pale yellow. Hind femur pale yellow to blackish brown.

Distribution. China, Japan, Korea, Mongolia, Russia (Far East), Central and Western Europe.

3.2. Biology of *Spathius verustus*

Previously, *S. verustus* was known to attack only two coleopteran hosts, including *M. alternatus* (Cerambycidae) and *Pissodes obscurus* Roelofs (Curculionidae) [65,66], but the present study identified *M. saltuarius* as a new host. *S. verustus* was found to be an idiobiont ectoparasitoid (Figure 3D). The wasp mostly parasitized the second instar of *M. alternatus* and the first instar of *M. saltuarius*. Host instar was determined from the average head capsule width (HCW), as per [67,68]: 1.3 mm for *M. alternatus* second instar larvae and 1.2 mm for *M. saltuarius* first instar larvae. Additionally, *M. alternatus* third (HCW 1.6 mm), fourth (HCW 2.3 mm), and fifth (HCW 3.0 mm) instar larvae were found parasitized. The number of progenies on a larva of each host was slightly different. Up to four parasitoids were found on a larva of *M. alternatus* and up to three parasitoids on a larva of *M. saltuarius*. The average number of the parasitoid larvae was 3.15 ± 1.64 on *M. alternatus* and 1.48 ± 0.62 on *M. saltuarius* (Table 1).

Table 1. Biological information of *Spathius verustus* by host.

Host	Larval Stages of Parasitized Hosts (*n*)	Ave. No of Parasitoids on a Host Larva	Ave. Pupation Period (Day)	Sex Ratio (♂: Total)
M. alternatus	2nd (7) 3rd (1) 4th (3) 5th (1)	3.15 ± 1.64	6.6 ± 1.39	0.16
M. saltuarius	1st (33)	1.48 ± 0.62	7.76 ± 3.06	0.37
	2nd (3)			

Additionally, the ANOVA test results showed only the relationship between host density and number of parasitized host larvae statistically significant, when the host was *M. alternatus*, although the sample numbers were too small for a consistent analytic result (Figure 4, Table 2). On the other hand, all the other relationships such as size of host larvae and number of *S. verustus* were not statistically significant.

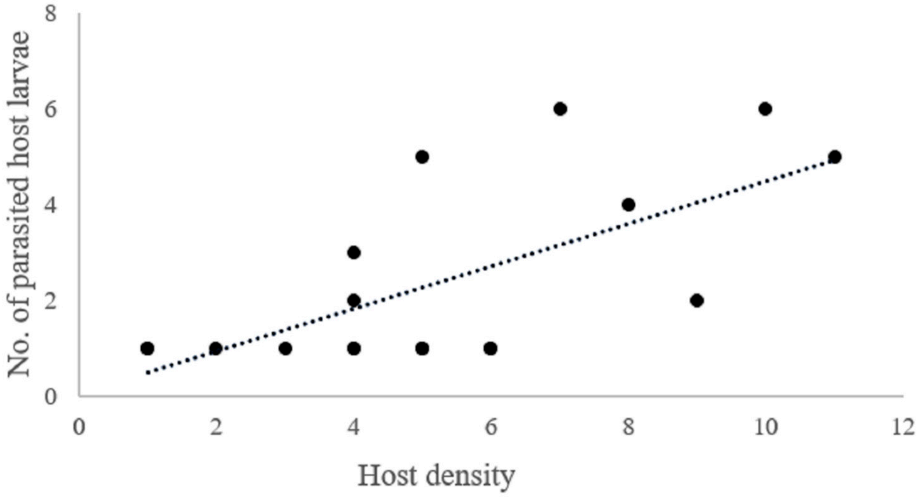

Figure 4. Relationship between host density (=number of host larvae per sentinel log) and number of parasitized *M. alternatus* larvae ($y = 0.4445x + 0.043$, $r = 0.66$, $df = 34$, $t = -1.64$, $p < 0.002$).

Table 2. Correlation analysis of host characteristics and *Spathius verustus* ecology in 2016 and 2017.

Host	Variable	*n*	*r*	*df*	*t*	*p*
M. alternatus	Size of parasitized host larvae × no. of *S. verustus*	12	0.35	10	1.16	0.27
	Size of parasitized host larvae × size of female *S. verustus*	25	0.26	23	1.3	0.21
	Host density × sex ratio	10	−0.55	8	−1.87	0.1
	Host density × no. of parasitized host larvae	18	0.66	16	3.56	0.002 **

Table 2. *Cont.*

Host	Variable	*n*	*r*	*df*	*t*	*p*
M. saltuarius	Size of parasitized host larvae × no. of *S. verustus*	36	−0.27	34	−1.64	0.1
	Size of parasitized host larvae × size of female *S. verustus*	57	0.09	55	0.6	0.55
	Host density × sex ratio	29	−0.27	27	−1.44	0.16
	Host density × no. of parasitized host larvae	45	0.17	43	1.11	0.27

** $p < 0.01$.

At the time of dismantling the sentinel logs, the early instar larvae of the parasitoid were white, and as they developed into mature larvae, fat bodies were distributed inside the body. The body size of fully matured larvae, with numerous fat bodies distributed rather sparsely throughout the body, was approximately 2–3 mm (Figure 3D). They then detached themselves from the body of the host before pupation. They spun silks and formed cocoons in the tunnel made by the host larvae. Upon this time, the host larvae lost all their body fluid and completely shrunk, with only the head remaining (Figure 3E). The parasitoid pupa was an exarate pupa, which was first white and then gradually turned darker over time (Figure 3F). The pupal stage lasted about 6.6 ± 1.39 d on *M. alternatus*, and 7.76 ± 3.06 d on *M. saltuarius* at room temperature 25 ± 3 °C (Table 1).

3.3. Indoor Oviposition Observation of *S. verustus*

In simple oviposition tests, *S. verustus* did not respond at all to either the exposed host larva or two types of wood sandwiches, with or without a host oviposition scar, even after several hours of exposure. On the other hand, the parasitoids exposed to a whole sentinel log approached certain areas within a few minutes after the exposure. A few of them were observed to have gathered in a certain area starting antennal drumming for a few minutes, then lowered their abdomen to start drilling (Figure 2D). After about ten minutes of drilling, the parasitoids started ovipositor probing as if trying to locate an exact or suitable point for oviposition and then put the ovipositor down and stayed still for a while. After this, the female withdrew its ovipositor completely and left from the site.

3.4. Parasitism Rates of *S. verustus* from the Field Surveys in 2016 and 2017

In 2016, among the survey sites, the average parasitism rate of Jinju was higher than Geoje and Suncheon and was statistically significant (Figure 5A, Table 3). The average parasitism rate by tree height was higher at 1.8 m than at 0 m, but there was no statistical significance (Figure 5B, Table 3).

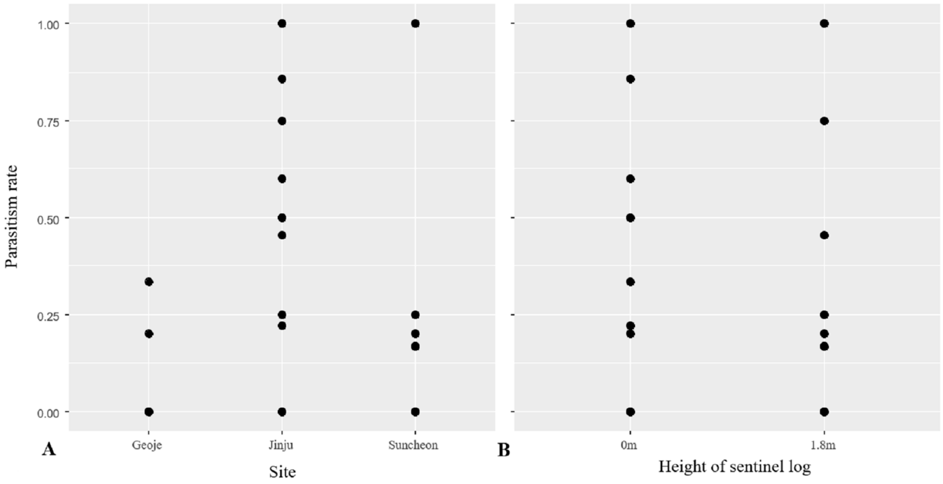

Figure 5. Scatter plot of *Spathius verustus* in 2016: (**A**) relationship between site and parasitism rate; (**B**) relationship between height of sentinel log and parasitism rate.

Table 3. Parasitism rate analysis of *Spathius verustus* by ecological variables using GLMM in 2016 and 2017.

Year	Variable	Parameter	Mean (%) ± SD	*n*	*df*	*p*
2016	Site	Geoje Suncheon Jinju	1.66% ± 6.61 5.59% ± 18.2 17.62% ± 31.26	96 †	2	4.279×10^{-12} ***
	Height of sentinel log	0 m 1.8 m	7.75% ± 21.18 8.83% ± 23.33		1	0.94
2017	Site	Gapyeong Yangpyeong Hongcheon	0.68% ± 0.03 1.25% ± 0.04 3.01% ± 0.09	480 †	2	0.001501 **
	Height of sentinel log	0 m 1.8 m	1.2% ± 0.04 1.4% ± 0.05		1	0.38
	Forest depth	0 m 10 m 20 m 30 m 40 m	1.43 ± 0.05 0.7% ± 0.03 0.89% ± 0.03 1.6% ± 0.04 1.91% ± 0.04		4	0.52
	Session	1st (5/17–31) 2nd (6/1–15) 3rd (6/15–30) 4th (7/1–15)	3.35% ± 0.07 0.28% ± 0.01 0.64% ± 0.02 2.12% ± 0.06		3	2.849×10^{-7} ***

** $p < 0.01$, *** $p < 0.001$. † total number of sentinel logs used for the survey.

In 2017, among the survey sites, the average parasitism rate of Hongcheon was higher than Yangpyeong and Gapyeong, and it was statistically significant (Figure 6A, Table 3). The average parasitism rate by height of sentinel log was higher at 1.8 m than at 0 m, but there was no statistical significance (Figure 6B, Table 3). The average parasitism rate (0 m, 10 m, 20 m, 30 m, 40 m) appeared to increase with forest depth, but it was not statistically significant (Figure 6C, Table 3). The average parasitism by session was high in the order of second, third, fourth, and first, and it was statistically significant (Figure 6D, Table 3). In addition, as a result of comparing the parasitism rate of *M. alternatus* and *M. saltuarius* as the hosts of *S. verustus*, the parasitism rate of *M. alternatus* was high, which showed a statistical significance ($p < 0.01$).

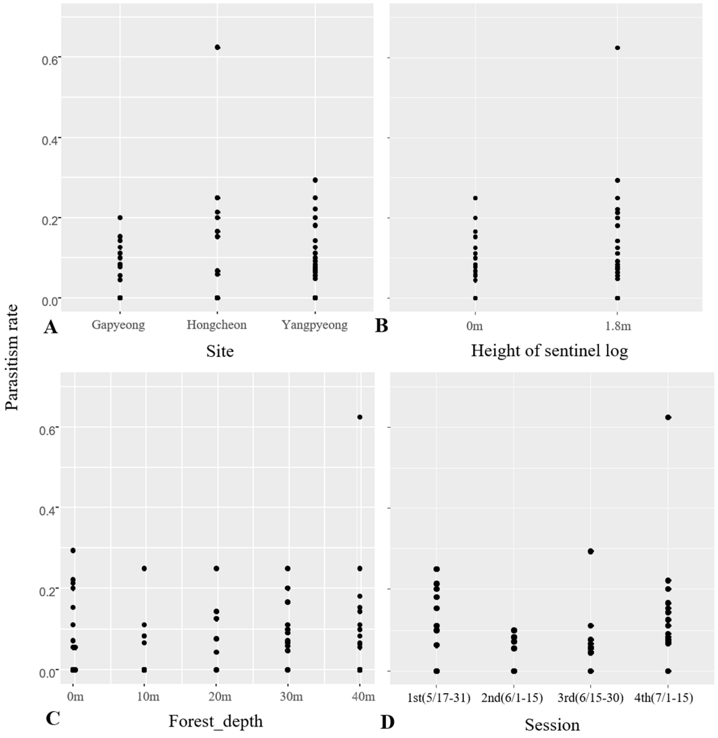

Figure 6. Scatter plot of *Spathius verustus* in 2017: (**A**) relationship between site and parasitism rate;

(**B**) relationship between the height of sentinel log and parasitism rate; (**C**) relationship between forest depth and parasitism rate; (**D**) relationship between session and parasitism rate.

4. Discussion

4.1. Indoor Oviposition Observation of S. verustus

Spathius verustus females were observed to display a series of ovipositional behaviors such as antennal drumming, drilling, and oviposition only when exposed to sentinel logs. This may indicate that *S. verustus* also responds to the same or similar ovipositional cues as *S. agrilis* did in a previous study [58]. According to them, *S. agrili,* used as the biological control of *A. planipennis*, the emerald ash borer, was reported to be attracted by the volatile substance from the leaves of ash trees when harmed by *A. planipennis* and the vibration generated when the beetle larvae gnawed the tree with its mandibles. Then, this may explain the reasons why *S. verustus* females did not respond to the hosts in the other conditions: (1) the parasitoids females were not attracted to naked hosts since no chemical cues are expected to be released and act as kairomone [69], and (2) the host larvae could scratch the wall to make a noise, but its body was not tightly fixed in place due to the space in the bark sandwich and was not able to generate vibrations strong enough to attract *S. verustus.* As an exact oviposition cue of *S. verustus* was not identified at the present time, it will be ideal to supply whole sentinel logs upon mass-rearing until an alternative host or artificial diet, which is more convenient than a whole sentinel log, is identified or developed.

4.2. The Ecological Relationship between the Host and S. verustus

There was no statistical significance in the relationship between the size of host larvae and the number of *S. verustus* in 2016 and 2017. Häckermann et al. [70] reported that the clutch size of parasitoids was not influenced by the host size. This suggests that the number of eggs was not proportional to the larval size of the host (Table 2). Nicol and Mackauer [71] reported that the host size did not affect parasitoid size. However, the size of female *S. verustus* was affected by the size of host larvae in 2016 and 2017 (Table 2). This will require further investigation with larger samples. Studies of the relationship between the sex ratio of *Bracon hebetor* and the density of the host have shown that as the host density increases, the male ratio tends to decrease and the female ratio tends to increase [72]. In 2016 and 2017, the relationship between host density and the sex ratio of *S. verustus* was similar to the result of Jamil et al. [72] but was not statistically significant (Table 2). This can be addressed by increasing the sample size in future studies. We observed a relationship between host density and the number of parasitized host larvae in 2016 and 2017. These results suggest that the number of parasitized host larvae increases with an increase in host density [73]. However, only the 2016 results are statistically significant, and further study on *M. saltuarius* is needed due to the small sample size (Table 2).

4.3. In Spatial Effect of Parasitism Rate of S. verustus According to Environmental Variables

In 2016 and 2017, parasitism rates for *S. verustus* were investigated in three regions, respectively, in the northern and southern regions of South Korea. Although the host was different, there was a statistically significant difference in the parasitism rate by region (Table 3). This may be related to the difference in host density among the sites. According to Furlong and Zalucki [74], the density of parasitoids changes with the density of the host. However, there has not been any study on host density in infected or healthy pine forests in the country. Therefore, further research is needed to investigate the density of the host and other factors so as to determine which factor is the cause.

During the investigation period, installation heights of sentinel logs were both 0 and 1.8 m, and the installation by forest depth was divided into 5 stages (0 m, 10 m, 20 m, 30 m, 40 m) from the edge to the inside of the forest in only 2017. However, the parasitism rate of *S. verustus* was not affected by installation height and forest depth. Chung et al. [75] investigated the within-tree distribution of larval entrance of *M. laternatus* and reported that larvae of *M. laternatus* were distributed most at 6 to 7 m and at least 0 to 3 m height of Korean red pines. This may suggest that the parasitism of *S. verustus* by sentinel log height was not statistically significant because the sentinel logs were installed at a height lower than where the hosts might have mostly been distributed in a tree. According to Ma et al. [76], when the density of *M. alternatus* was investigated using a pheromone trap outdoors, it was highest at 0 to 50 m from the forest edge toward the center. In the present study, all of the sentinel logs were installed within the forest depth range where the vector might be mostly distributed. Therefore, the parasitism rate did not show differences along the forest depth.

In 2017, the parasitism rate of *S. verustus* was affected by session as the emergence time of the host (Table 3). The parasitism rate of the 1st (5/17–31) and 4th (7/1–15) is higher than the 2nd (6/1–15) and 3rd (6/15–30) of the session. There are about 40 days between the 1st and 4th parasitism peaks. It is thought that the occurrence of a natural enemy is closely related to the life cycle of the host. According to Han et al. [77], the first emergence time of *M. saltuarius* is in early May and lasts until mid-June in South Korea. The life cycle *S. agrili*, which is a braconid parasitoid of the emerald ash borer, takes about 40 days from one generation to the next [78]. If *S. verustus* has a life cycle similar to that of *S. agrili*, the interval between two parasitism peaks in the 2017 survey result could be due to the developmental period of *S. verustus*. In addition, *S. verustus* appears to prefer *M. alternatus* to *M. saltuarius*, which shows which host to use for future indoor breeding.

4.4. Possibility of *S. verustus* as a Biological Control Agent on the Insect Vectors of PWN

Developing a biological control project requires investigating natural enemies, especially specialists, their availability in nature, biological traits, etc. The present study can be considered as the very first step, in finding natural enemies and exploring basic biological traits, toward developing biological control against the spread of PWD.

Two insect vectors in South Korea had never been problematic before the PWN invasion, but now they are a key factor in the spread of PWD. It would be another viable biological control option to cut the link between the *Monochamus* vectors and PWN by utilizing natural enemies of the vectors so as to slow down or restrain the spread of PWD. In regard to restraining PWN transmission, there is ongoing research on chemical interactions among PWN, insect vectors, and host trees to break the link in the transmission from the infected wood to the insect vectors and later from the insect vectors to the healthy host trees during feeding [69,79].

Spathius verustus appears to be distributed nationwide, from northern to southern regions, and up to date, the wasp seems to be a generalist but with a narrow range of host species, now including two Korean native *Monochamus* species. This trait alone may make the parasitoid a potential candidate as a biological control agent against both of the PWN insect vectors; controlling two pest species with one natural enemy species anywhere in the country would be very cost-effective.

Secondly, the parasitoid was most parasitic on the second instar larva of *M. alternatus* and the first instar larva of *M. saltuarius*. This may be explained by the ovipositor length, about 2 mm, which means that the parasitoid cannot parasitize host larvae living deeper than 2 mm from the bark. Previously, Pershing and Linit [80] reported that the fourth instar larvae of both *Monochamus* species move into the sapwood area, and from then on, the probability of being attacked by their natural enemies would be significantly reduced, which makes it difficult to control them. Therefore, using the parasitoid to attack the early-stage larvae would be an effective measure to control the insect vectors.

Thirdly, the parasitoid can be considered as gregarious, laying an average of 3.15 ± 1.64 eggs on the larvae of *M. alternatus* and more than 1.48 ± 0.62 eggs on *M. saltuarius*, which is advantageous in terms of mass-rearing of any parasitoid. Compared to this, *S. agrili*, a biological control agent on the emerald ash borer, has been reported to lay an average of eight eggs on a host [81]. Although the number of eggs laid by a parasitic wasp is different depending on host size, it can be a great advantage in increasing the number of *S. verustus* as long as host larvae are well managed, at the instar stages preferred by the parasitoid, during mass-rearing [82]. Further indoor tests are required to investigate the accurate life cycle of *S. verustus* to figure out biological characteristics such as developmental period, life span, daily fecundity, and sex ratio.

Lastly, the flight period of the parasitoid appears to coincide with the flight period of the two *Monochamus* species, as the parasitoid constantly parasitized the hosts throughout the survey period. Therefore, the parasitoid can be augmented into a certain area during the peak flight period of the vectors, when their oviposition period can be assumed to be from mid-May to late June according to Han et al. [77].

4.5. Compensate the Defect and Future Study

In this study, the large difference in the parasitism rate in the two vectors and the parasitism rate by region needs to be addressed in the future. According to Choi et al. [18], the intraspecific competition among the parasitic wasps resulted in an increase in the density of the parasitic wasps as the density of the vectors decreased over time. Referring to this and considering the history of the area affected by PWD, some regional characteristics may have affected the parasitism rate. For example, the Jinju site has been an area devastated by PWD for the longest time next to Busan where PWD was first identified [83]. In addition to that, we were told that the Jinju site of the present study has never been treated with aerial spray except for nematicide injection for experimental purposes. Insecticide spraying reduces the vector's density, as well as the parasitoid's density [84]. Therefore, this area seems to have been stably accommodating numbers of *S. verustus* parasitic to *M. alternatus* [85]. In future studies, it is necessary to investigate other infested areas with parasitoids.

Lastly, to use *S. verustus*, which has been identified to be parasitic to the vectors of PWN, as a biological control agent, it is necessary to address several limitations. For example, the host range of *S. verustus* requires further research. If this species is only parasitic to the wood-boring beetles that only inhabit live pines, it would be a highly interesting option in terms of forest protection. Unlike the chemical or physical control methods, such a control method for various wood-boring insect pests would enable pest control without a great impact on the environment [86]. Recently, with increased negative awareness of chemical control agents and continuous reports on environmental risks such as the harm to honeybees [87], it is critical to explore, select, and utilize the means of biological control for large-scale forests. Therefore, continuous research on other natural enemies, including parasitoids such as *S. verustus*, as well as predators is required.

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