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Pollination by Three Species of Stingless Bees (Hymenoptera: Meliponini) Increase Seed Set of Mustard (*Brassica rapa* L.: Brassicaceae)

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ABSTRACT

Pollination and fertilization are critical factors in horticultural cultivation to increase seed production. In this study, we measured the visiting activity and pollination of three species of stingless bees, i.e., *Tetragonula laeviceps*, *Lepidotrigona terminata*, and *Heterotrigona itama* in mustard (*Brassica rapa*). Visiting activity of stingless bees were observed in mustard plants caged by an insect screen. The observations were done visually per hour, starting from 07:00 until 16:00, using the focal sampling method. The visiting activities observed were flower handling time, foraging rate, and total visit per plant. We also measured the pollen load of each stingless bee species. Results showed the highest foraging rate occurred in *H. itama* (28.38 flowers/5 minutes), followed by *T. laeviceps* (27.88 flowers/5 minutes), and *L. terminata* (27.85 flowers/5 minutes), respectively. The longest flower handling time was found in *H. itama* (40.15 seconds), followed by *T. laeviceps* (35.25 seconds), and *H. terminata* (34.84 seconds). Whereas, the highest total visiting time occurred in *H. itama* (40.10 minutes), followed by *T. laeviceps* (37.47 minutes), and *L. terminata* (36.76 minutes). *Heterotrigona itama* has a highest pollen load (36,650 pollen grains), followed by *L. terminata* (26,940 pollen grains), and *T. laeviceps* (9,700 pollen grains), respectively. Pollination by the stingless bees increase 27-40% the number of pods per plant, 32-72% the number of seeds per pod, 32-54% of seed weight per pod, and 18-36% of seed germination of mustard.

1. Introduction

In the world, stingless bees (Apidae: Meliponinae) are distributed in tropical and subtropical areas (Pauly *et al.* 2013). About fifty species of stingless bees were recorded (Sakagami 1971). Stingless bees are highly social insects with diverse lifestyles (Camargo and Roubik 1991) and a major visitor of various plant species in agricultural lands (Slaa *et al.* 2006). The visiting activity of stingless bees has been reported previously. Tangmitcharoen *et al.* (2006) reported *Tetragonilla collina* as important in teak pollination and has a high visitation rate (73.95%) and increased activity at 10:00-12:00. The activity of pollinating insects on the teak flower is associated with the number of pollen and nectar secretions. In *Brassica juncea*, Kunjwal *et al.* (2014) also reported *T. laeviceps* visited 3.67 flowers/minute. The foraging rate of pollinating bees is strongly influenced by microclimate and the quality of pollen and nectar (Klein *et al.* 2004).

The high foraging activity of bees is indicated by the number of pollen attached to the body (Ramalho *et al.* 2009). Inoue *et al.* (1985) reported in disturbed forest areas of Sumatera, three species of stingless bees (*Tetragonula minangkabau*, *Trigonella moorei*, and *Heterotrigona* sp.) collected pollen as many as 10-20%, 70-80%, and 10%, respectively. The species actively collected pollen in the morning, but in *H. itama*, the peak of nectar collection occurs mid-day. Wulandari *et al.* (2017) reported in kale, *T. laeviceps* collected more pollen (8,125 pollen grains) in caged

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plants compared to uncaged plants (3,000 pollen grains). During foraging, competition for mustard flower resources occurred between social and solitary bees (Atmowidi *et al.* 2007).

The role of stingless bees in pollinating crops has been reported previously. Klein *et al.* (2003) reported pollination of coffee by *L. terminata* increased by 84% of fruit set. In Australia, pollination by stingless bees increases by 36% of mango (*Mangifera indica*) (Anderson *et al.* 1982). In Indonesia, stingless bees also were reported as an effective pollinator of *Jatropha curcas* (increased production by more than 40%) (Kasno *et al.* 2010), and strawberry (increased 48% of fruit set) (Widhiono *et al.* 2012). In kale plants, *T. laeviceps* pollination increases by 231% of pod number, 48% of seed number per pod, 204% of seed weight, and 24% of seed germination (Wulandari *et al.* 2017).

Three species of stingless bees used in this study were *Tetragonula laeviceps* L., *Lepidotrigona terminata* (Smith 1878), and *Heterotrigona itama*. These species have different body sizes. *Tetragonula laeviceps* has a small body size (3.44-4.88 mm in length), *L. terminata* has medium body size (about 5 mm in length), and *H. itama* has a large body size (about 6.15 mm in length) (Sakagami 1978). *Tetragonula laeviceps* is characterized by tegument polished, minutely punctate, mandible with two small teeth, malar area shorter than the diameter of the antennal flagellum, and abdomen slightly narrower than thorax, rather flat, and anterior terga polished. *Lepidotrigona terminata* is characterized by the tessellation of the integument, particularly on the head and thorax, and by the yellow ring outlining the scutum. Mandibles with two small teeth, malar area medium, equal to the flagellar diameter, and abdomen narrower than thorax, elongate, and in three first segments mainly smooth. *Heterotrigona itama* is characterized by a single tooth or denticle on the mandible, tegument shiny, delicately and finely punctate, malar area as long as or longer than the flagellar diameter, and abdomen narrower than thorax, elongate, its transverse section sector-shaped, and in first three terga shiny (Smith 2012).

Mustard (*Brassica rapa* L.) is an important crop in Indonesia and Asia. Flowers of the species are formed in the stem elongates after the leaf stop growing. The flowers have small size. The mustard needs pollinators matching the flower size, such as stingless bees, to maximize the fruit set. However, the biology of stingless bees as pollinator is not fully known. Therefore, this study aimed to study the visiting activity and the role of three stingless bees, namely *T. laeviceps, L. terminata,* and *H. itama* in pollination and fruit set of mustard.

2. Materials and Methods

2.1. Study Sites

This study was conducted from August to December 2015 in the Cikabayan Experimental Farm of IPB University in Bogor, West Java, Indonesia. The measurements of pollen load and data analysis were conducted in the laboratory of Animal Biosystematics and Ecology, Department of Biology, IPB University.

2.2. Preparation of Plants

The seeds of mustard were sown in a tray with media made by organic fertilizer. Fourteen days after sowning, 250 plants were planted in the field using a zig-zag pattern with a distance of 25 cm x 25 cm. The organic fertilizer was used once a week. We controlled the pest manually without any pesticide application.

2.3. Selection of Stingless Bee Colony

All stingless bee colonies used in the study, i.e., *T. laeviceps, L. terminata*, and *H. itama* were obtained from Rangkasbitung, Lebak District, Banten Province. A good colony was used with criteria having a large number of individuals, a healthy colony, and the form of nest structure was perfect.

2.4. Application of Stingless Bees to Mustards Pollination

Two hundred and fifty plants were planted in the Cikabayan experimental farm of IPB University. Fifty plants were caged (size 5 m x 3 m x 2 m) using an insect screen, and four cages in similar size were set up, and each cage contained 50 plants. Before flowering, one colony of stingless bee species was put in the cage. Fifty plants caged by screen, without a stingless bee colony, were used as a control. We also used open fifty plants to observe the effect of natural pollinators in mustard pollination.

2.5. Observation of Visiting Activity of Stingless Bees

The visiting activities of the stingless bee on mustard flowers were observed visually every hour using the focal sampling method (Martin and Bateson 1986), starting from 07:00 until 16:00 on sunny days for ten days. The visiting activities observed included foraging rate, flower handling time, and total visit time per plant. The foraging rate was measured by the number of flowers visited per 5 minutes. The flower handling time was measured by the time used by stingless bees per flower. Whereas the total visit time was measured by the total times used by stingless bees per plant.

2.6. Measurements of Pollen Load

Pollen load on the body of each stingless bee was measured. One individual of each stingless bee species that returned to the hive was caught by sweep net. The sample was put in a microtube contained 70% ethanol and glycerol with a composition 4:1 (Dafni 1992). Then, the microtube was centrifuged at 300 rpm for 5 minutes. After being centrifuged, 0.1 ml of pellet that contained pollen was shed at the hemocytometer and observed under a compound microscope embedded with the camera. The number of pollen loads by each species of stingless bee was counted based on the formula: N2 = (N1.V2)/V1, where V2 = volume of sample solution (0.1 ml), N1 = number of pollens in four quadrants of hemocytometer, V1 = volume of four quadrants of hemocytometer (0.4 mm^3). The measurement of the pollen load of each stingless bee species was replicated as many as ten times.

2.7. Measurement of Mustard Yields

The yields of mustard were measured two months after planting. Randomly, ten plants of each treatment were measured, i.e., plant height, pod number per plant, seed number, and weight per plant. Seeds germination also were measured with five times replications.

2.8. Data Analysis

The plant height, pod number per plant, seed number and weight per plant, and seed germination were compared and analyzed using analysis of variance (ANOVA) followed by the Tukey test (α = 0.05). Data of the visiting activity of stingless bees were shown in the line graphics, while the pollen load was shown in the bar chart. The number of pollens carried was correlated with the individual weight of the stingless bee.

3. Results

3.1. Visiting Activity and Pollen Load

The current study showed the visiting activity of each stingless bee species varied. The highest visiting activity occurred from 11:00 to 12:00. The highest foraging rate occurred from 15:00-16:00, i.e., *T. laeviceps* (27.88 flowers/5 minutes), *L. terminata* (27.85 flowers/5 minutes), and *H. itama* (28.38 flowers/5 minutes) (Figure 1). The longest flower handling time of *T. laeviceps* and *L. terminata* occurred in 11:00-12:00 (35.25 seconds/flower, 34.84 seconds/flower), while in *H. itama* occurred in 08.00-09:00 (40.15 second/flower) (Figure 2). Whereas, the longest total visit time per plant of *T. laeviceps, L. terminata,* and *H. itama* occurred in 11.00-12:00 (37.43 minutes, 36.76 minutes, 40.10 minutes, respectively) (Figure 3). The current study showed *H. itama* had a highest pollen load (36,650 pollen grains), followed by *L. terminata* (26,940 pollen grains), and *T. laeviceps* (9,700 pollen grains), respectively (Figure 4).

3.2. Seeds Set

The study showed that pollination by stingless bees increases the pod numbers per plant and seed number, and weight per pod. The yields of mustard pollinated by three stingless bee species were higher than open plants (pollinated by natural pollinators) and control plants (without the stingless bee). Pollination by *T. laeviceps, L. terminata*, and *H. itama* increases 27-40% of pod number per plant, 32-75% of seed number per pod, and 14-128% of seed weight of mustards. Pollination by stingless bees also increases seed germination. Pollination by *H. itama* increases the highest seed germination (36%), followed by *L. terminata* (30%), and *T. laeviceps* (18%), respectively (Table 1).

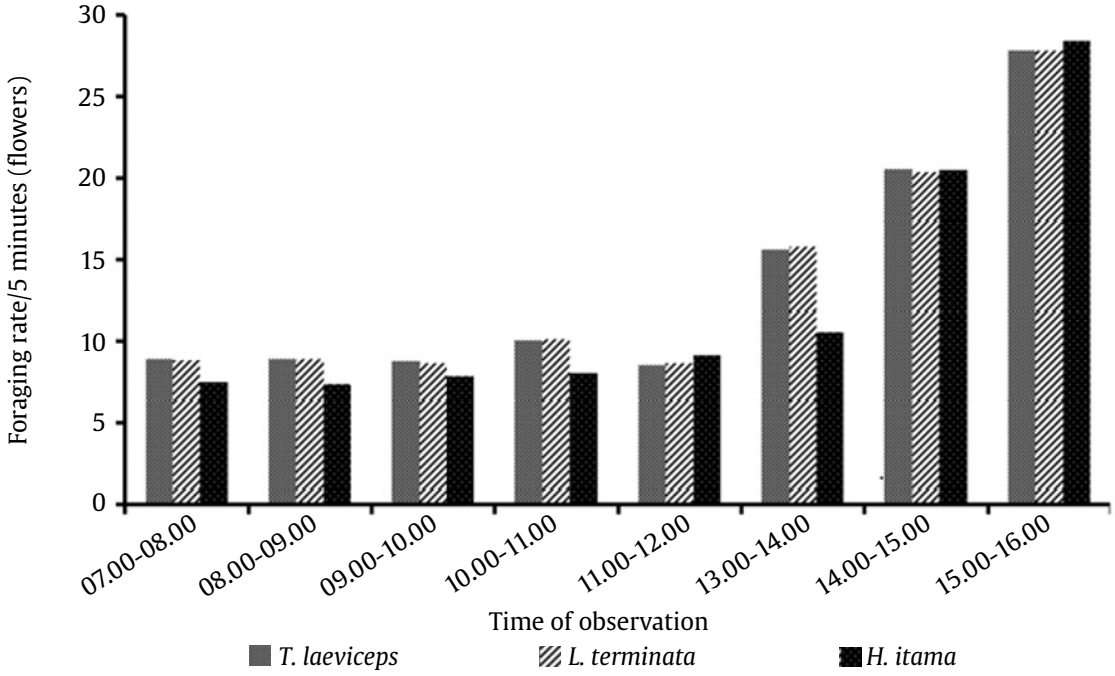

Figure 1. Foraging rate of three species of stingless bees: *T. laeviceps*, *L. terminata*, and *H. itama* on mustard flowers based on block time observation

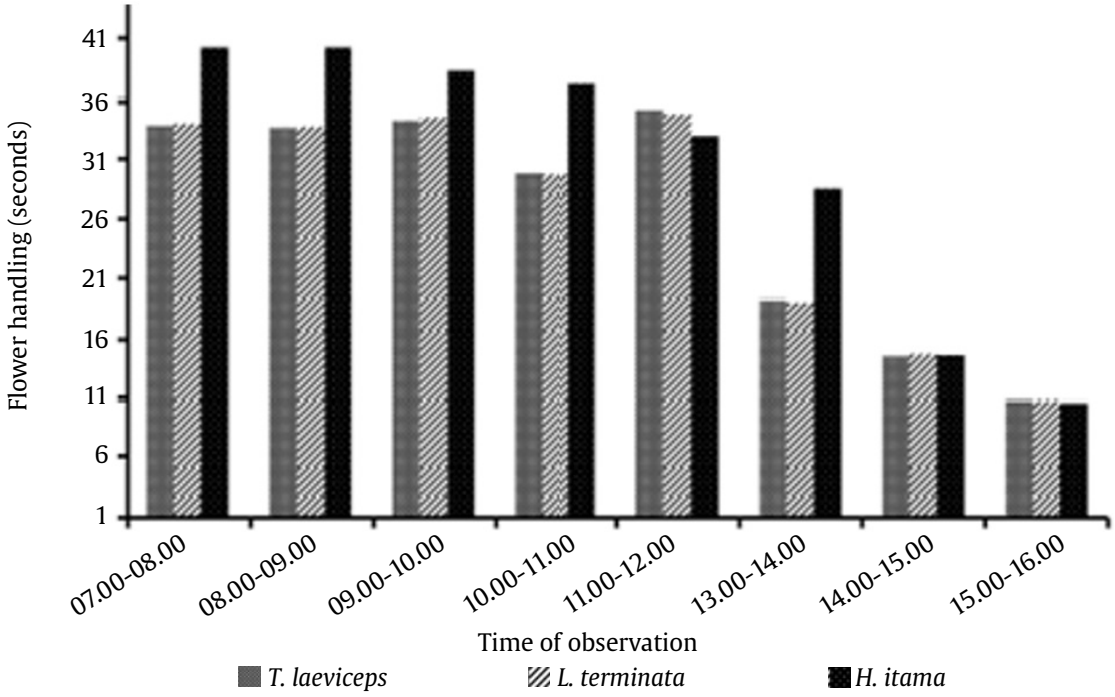

Figure 2. Flower handling time of three species of stingless bees: *T. laeviceps*, *L. terminata*, and *H. itama* on mustard flowers based on block time observations

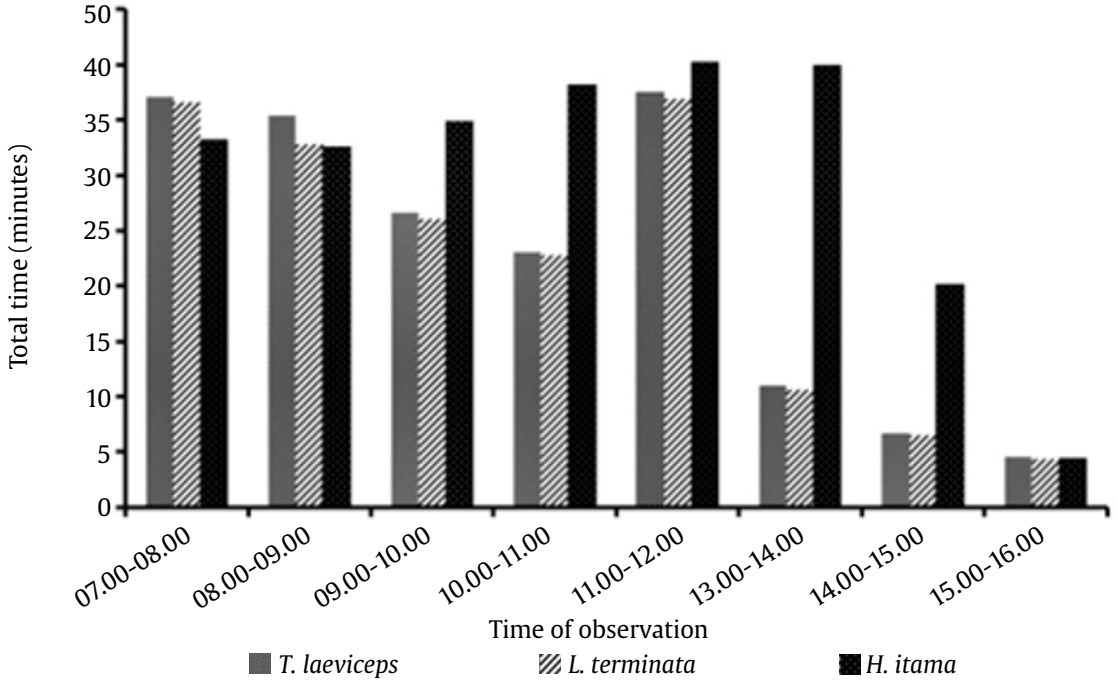

Figure 3. Total visiting time per plant of three species of stingless bees: *T. laeviceps, L. terminata*, and *H. itama* on mustard flowers based on block time observations

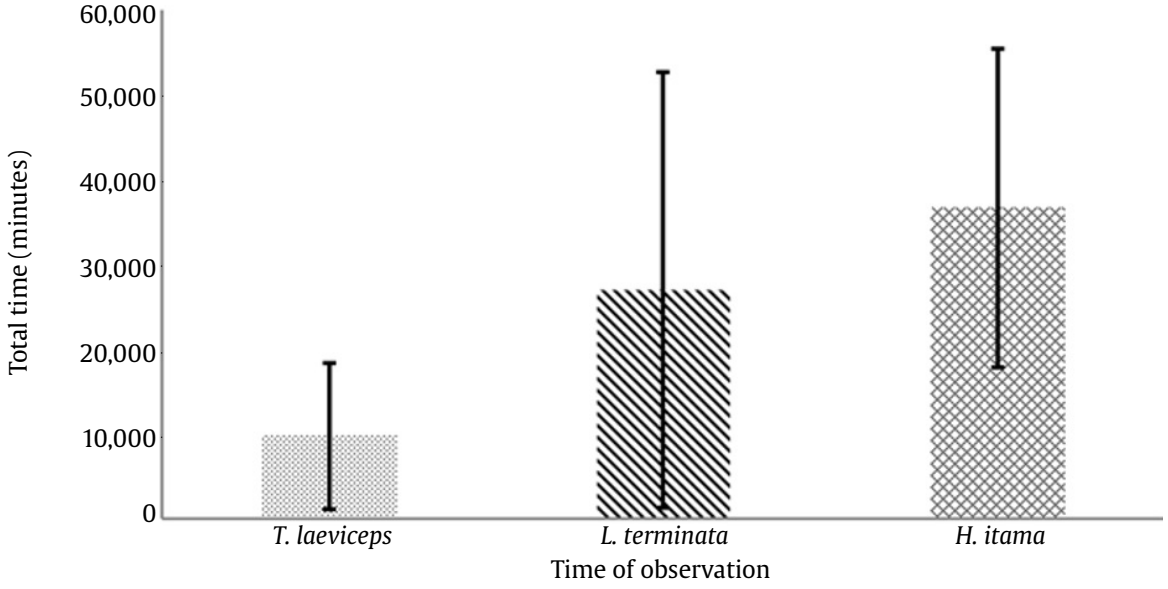

Figure 4. The number of pollen load on T. *laeviceps, L. terminata*, and *H. itama*. Bars showed standard deviation

Table 1. An increasing yields of mustard plants pollinated by *T. laeviceps, L. terminata*, and *H. itama* compared with opened- and control plants

Plant height and yields	Mustard yields ± standar deviation					Increase (%)		
	Opened plants	Closed plants (control)	Closed plants with *T. laeviceps*	Closed plants with *L. termi*nata	Closed plants with *H. itama*	Closed plants with *T. laeviceps*	Closed plants with *L. termi*nata	Closed plants with *H. itama*
Plant height (cm)	145±5.18^{c}	30.00±10.14^{c}	131.00±10.20^{c}	137.00±9.97^{b}	130.00±10.42^{b}	-	-	-
Number of pods per plant (pods)	96.00±31.00^{b}	113.00±6.14^{b}	145.00±1.00^{b}	154.00±23.24^{c}	159.00±20.12^{c}	27	36	36
Number of seeds per pod (seed)	7.80±1.98^{a}	6.0±1.69^{a}	7.80±1.98^{b}	9.30±3.80^{a}	10.30±3.09^{a}	32	55	55
Seed weight per plant (gr)	3.68±0.30	2.53±0.20	3.68±0.30	5.35±0.88	6.14±1.18	121	128	128
Seed germination (%)	68	66	78	86	90	18	30	30
Seed harvest time (months)	± 2	± 3	± 2	± 2	± 2	-	-	-

The different letters on the same line showed different values based on the analysis of variants of ANOVA, followed by Tukey test ($\alpha = 0.05$)

4. Discussion

4.1. Visiting Activity of Stingless Bees

The visiting activity of stingless bees started in the morning until evening, and the peak activity occurred at about mid-day. In average, the foraging rate of three stingless bees ranged from 12.40 to 13.65 flowers/5 minutes. The current study showed the foraging rate of stingless bees were lower than other bee species, i.e., *Xylocopa confusa* (33.8 flowers/minute), *Amegilla cyrtandrae* (27.08 flowers/minute) in tomato (Indraswari *et al.* 2016), *X. confusa* (12.55 flowers/minute), and *Megachile conjuncta* (9.61 flowers/minute) in cucumber (Hasan *et al.* (2017). The foraging rate of stingless bees in this study was similar with *Ceratina cognata* (2.24 flowers/minute) in tomatoes (Indraswari *et al.* 2016), *Ceratina* sp. (1.79 flowers/minute), and *Nomia* sp. (1.40 flowers/minute) in cucumber (Hasan *et al.* 2017). In the afternoon, the foraging rate of three stingless bee species slightly increased, i.e., 27.88 flowers/5 minutes (*T. laeviceps*), 27.85 flowers/5 minutes (*L. terminata*), and 28.38 flowers/5 minutes (*H. itama*). This activity is probably due to the limited flower resources (nectar and pollens). Flower handling time of three stingless bee species in this study (26.43-30.31 seconds/flower) was similar to *C. cognata* (26.9 seconds/flower) in tomato (Indraswari *et al.* 2016) and *Ceratina* sp. (33.36 seconds/flower) in cucumber (Hasan *et al.* 2017). A similar result also was reported by Ruslan *et al.* (2015) that *T. laeviceps* has a higher visit frequency (27.47 seconds/flower) than *A. cerana* (6.22 seconds/flower) in mustard. Results showed that the total visit time of three stingless bee species (21.91-30.28 minutes/plant) was shorter than the total visit time of *C. cognata* (106.57 seconds) in tomato (Indraswari *et al.* 2016), *Ceratina* sp. (52.15 seconds), and *Nomia* sp. (79.57 seconds) in cucumber (Hasan *et al.* 2017).

The small body size of the stingless bee is suitable for mustard flowers. Food sources are available in caged plants, and no interspecific competition occurs during foraging. On the contrary, other insect pollinators were found in the open plants, such as *Apis cerana, Ceratina* sp., and *Xylocopa* sp. that competed with stingless bees. Atmowidi *et al.* (2007) also reported that competition and sharing resources occurred between stingless bees and other bees during foraging. Raju and Ezradanam (2002) also showed competition between pollinator insects and other pollinators for food resources. Sharing resources in insect flowers-visiting also was shown in different time activities, i.e., large-body size bees forage in the morning and a small-body size forage during the day. Our observations showed that bees with large-body sizes, such as *Xylocopa* sp. was not effective as a pollinator in mustard with a small flower.

4.2. Pollen Load

The number of pollen in the corbicula of stingless bees showed the species actively forage on mustard flowers. Current study showed the pollen load on three stingless bee species varied. *Heterotrigona itama* has a higher pollen load than *L. terminata,* and *T. laeviceps* (Figure 4). The higher pollen load in *H. itama* related to its body size (6.15 mm in body length) (Inoue *et al.* 1985). The body size of *H. itama* is larger than *T. laeviceps* (3.44-4.88 mm in length) (Sakagami 1978) and *L. terminata* (5mm in length) (Jalil and Shuib 2014). Results showed that pollen loads on three stingless bee species was similar with *H. itama* (31,392 pollen grains), *L. terminata* (23,017 pollen grains), and *T. laeviceps* (8,015 pollen grains) collected at Ecology Park (Cibinong Science Center-Botanical Garden) of BRIN, Cibinong, Bogor, West Java (Pangestika *et al.* 2017). Pollen load of three stingless bee species in this study also were higher than in *X. confusa* (13,375 pollen grains), *A. cyrtandrae* (7,625 pollen grain), *C. cognata* (3,325 pollen grains) in tomato (Indraswari *et al.* 2016), *Ceratina* sp. (350 pollen grains) and *Megachile* sp. (125 pollen grains) in cucumber (Hasan *et al.* (2017). The small-body size of stingless bees causes it easier to visit more flowers. The high number of flowers visited can be observed by the number of pollen attached to the body. Anderson *et al.* (1982) stated that stingless bees are effective pollinators since they often move from flower to flower and load a large number of pollens (482 pollens). In a single trip, bees tend to visit the flowers repeatedly of the same plant species (Graham 1992).

4.3. Seed Set

The stingless bee pollination increased the number of pods, seeds number, and weight per plant of mustard. The increased yields of mustard pollinated by stingless bees were higher than those pollinated by natural pollinators and control plants (Table 1). The increasing seed set of mustards pollinated by *T. laeviceps, L. terminata*, and *H. itama* ranged from 121 to 142%. Similarly, Delaplane and Mayer (2000) reported insect pollinators increased the number of pods, seed number, and weight of *B. campestris*. In *B. rapa*, Atmowidi *et al.* (2007) also reported natural insect pollinators increased 178.8% of the pod number per plant, 98.2% of the seed number per pod, and 932.5% of the seed number per plant. In *B. campestris*, Khan and Chaudhary (1988) reported insect pollination increased the seed formation (11.2%) compared to self-pollination (10.24%). Pollination by stingless bees also increases the fruit production and seed germination of paprika (de Oliveira Cruz *et al.* 2005) and apples in Brazil (Viana *et al.* 2014). The current study showed seed viability of mustard pollinated by stingless bees increased 18-36% compared to control plants. The high crop yields in mustards showed that the stingless bees are effective pollinators due to cross-pollination. Stingless bees pollination also made a shorter harvest time (Table 1).

In conclusion, this study showed the importance of stingless bees as pollinators of various crops, especially plants with small-size flowers. Results showed stingless bees significantly increase the seed set of mustards. To increase the productivity of crop plants, stingless bees can be applied by farmers for the enrichment of natural pollinators.

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