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REVIEW

Development of Molecular Identification Methods for a Sciarid Fly, *Bradysia odoriphaga* (Diptera: Sciaridae)

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Abstract

Recently, damage to Welsh onion, carrot, and Chinese chive by *Bradysia odoriphaga* Yang & Zhang, 1985 was reported from Saitama and Gunma Prefectures, Japan. Molecular identification methods are required to differentiate this species from other domestic sciarid flies. First, a PCR method using species-specific primers was developed to discriminate this species from three other sciarid species, *Bradysia impatiens* (Johannsen, 1912), *Pnyxia scabiei* (Hopkins, 1895), and *Lycoriella ingenua* (Dufour, 1839). Second, the applicability of a nondestructive DNA extraction method for this species was determined for cases where further morphological identification was required. It could be used for the morphological identification of specimens in addition to PCR using species-specific primers and DNA barcoding. Third, the lower limit of the proportion of this species in bulk samples of sciarid flies was determined. In larvae and male adults, one individual of this species could be detected in bulk samples (up to 800 individuals) of other species by PCR using species-specific primers. Further, it was revealed that specimens on the monitoring traps in the field should be collected within 8 days, regardless of the season. By combining these methods, an efficient identification method for monitoring this species was established using PCR with species-specific primers.

Discipline: Agricultural Environment
Additional key words: bulk samples, monitoring, mtCOI, nondestructive DNA extraction, species-specific primers

Introduction

The dipterous family Sciaridae (black fungus gnats) consists of inconspicuous dark-colored nematoceran flies only 2 mm-3 mm long (Mohrig et al. 2012). The genus *Bradysia* (Diptera: Sciaridae) includes some important agricultural pests such as *Bradysia impatiens* (Johannsen, 1912) (Mohrig et al. 2012). In Japan, at least 19 cultural crops from 12 plant and fungal families have been reported as actual and potential hosts for this species (Sueyoshi & Yoshimatsu 2019).

In recent years, damage to Welsh onion and carrot by larvae of an unknown species of *Bradysia* has been reported in Saitama Prefecture, Japan (Plant Protection Office of Saitama Prefecture 2016). Similar damage to Welsh onion and Chinese chive by larvae of this species was also reported from Gunma Prefecture, Japan (Gunma Agricultural Technology Center 2017, 2019). Based on the molecular phylogenetic analysis conducted by Arimoto et al. (2018) and on further morphological examination, this species was identified as *Bradysia odoriphaga* Yang & Zhang, 1985 (Sueyoshi & Yoshimatsu 2019). In China, it has been reported that this species causes serious damage to Chinese chive, and that Welsh onion, garlic, cucumber, lettuce, and Chinese cabbage are also host plants for this species (Ye et al. 2017). To prevent an expansion of the distribution areas of this species, it is important to rapidly and accurately identify this species.

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Traditionally, sciarid species have been identified based on morphological characters, especially on the male genitalia (Mohrig & Menzel 2009). However, other sciarid species morphologically similar to *B. odoriphaga*, such as *B. impatiens* and *Pnyxia scabiei* (Hopkins, 1895), have been known to co-occur with *B. odoriphaga* in Welsh onion fields (Omata 2019). *Lycoriella ingenua* (Dufour, 1839) may also co-occur in the fields because this species is known as an agricultural pest in Japan (Sasakawa 1993). In addition, the family Sciaridae living in Japan may comprise many undescribed and unrecorded species with similar morphologies (Sasakawa 2014, Nakamura 2016). Therefore, these species may also be collected in the fields along with *B. odoriphaga*. Further, only immature stages may be collected from damaged host plants. In such cases, many samples must be individually reared to obtain male adults. This procedure is time-consuming and hinders rapid species identification. Such situations require molecular identification methods for species identification.

In this article, I introduce methods to identify *B. odoriphaga* using molecular markers. First, I discuss the use of species-specific primers for the identification of this species (Arimoto et al. 2018). Second, I examine a nondestructive DNA extraction method for this species that has been developed (Arimoto et al. 2020a) for cases wherein further morphological identification is required. Third, I determine the lower limit of the proportion of this species in bulk samples of sciarid flies (Arimoto et al. 2020b). Further, I describe the effect of the exposure period of adults on traps in fields on the PCR success rates (Arimoto et al. 2020c).

Molecular identification of *B. odoriphaga* using PCR with species-specific primers

Arimoto et al. (2018) designed a species-specific primer, BCOF1 (5′-TTCTCATTCAGGTGCATCAGTA-3′), for the identification of *B. odoriphaga* on mitochondrial cytochrome *c* oxidase subunit I (mtCOI) gene, a typical DNA barcoding region (Fig. 1). Used in conjunction with universal primers (LCO1490 and HCO2198; Folmer et al. 1994), *B. odoriphaga* adults and larvae can be discriminated from those of *B. impatiens*, *P. scabiei*,

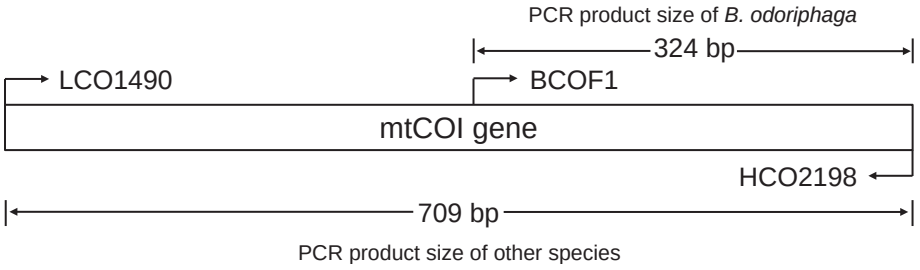

Fig. 1. Conceptual diagram of PCR product size for *Bradysia odoriphaga* and other species resulting from PCR analysis using species-specific primers

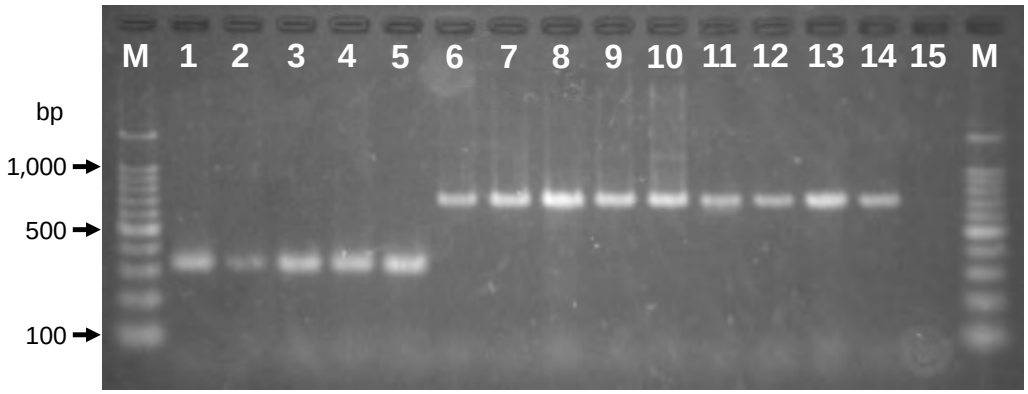

Fig. 2. Photograph of PCR-amplified products from the specimens of *Bradysia odoriphaga* (1-5), *Bradysia impatiens* (6-10), *Pnyxia scabiei* (11-12), and *Lycoriella ingenua* (13-14)
M: 100-bp ladder DNA size marker; 1-2: *B. odoriphaga* (adult), 3-5: *B. odoriphaga* (larva), 6-10: *B. impatiens* (adult), 11-12: *P. scabiei* (adult), 13-14: *L. ingenua* (adult), 15: negative control
(Modified from Arimoto et al. 2018)

and *L. ingenua* (Figs. 1 and 2). The size of the PCR products of *B. odoriphaga* was 324 bp, whereas that of *B. impatiens*, *P. scabiei*, and *L. ingenua* was 709 bp. These results indicate that this method is applicable for the rapid identification of *B. odoriphaga* collected during field monitoring.

Nondestructive DNA extraction method for *B. odoriphaga*

When undescribed or unrecorded species are collected during monitoring in Welsh onion fields, they may not be identified by DNA barcoding due to the lack of reference sequences in the International Nucleotide Sequence Databases. In addition, many different classifications have been applied to some species in the genus *Bradysia* (Sueyoshi & Yoshimatsu 2019). In such situations, to accurately identify species, it is necessary to examine the detailed morphological characters of the adults in addition to DNA sequence analysis. In addition, when many specimens are collected during monitoring in the field, the nondestructive DNA extraction method saves a lot of labor. Therefore, a nondestructive DNA extraction method for efficient DNA extraction and exact species identification of *B. odoriphaga* was examined (Arimoto et al. 2020a).

One hundred specimens of each developmental stage of *B. odoriphaga* were used. Each specimen was incubated with 50 µL of PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, CA, USA) for 20 min at 100°C. After removing each specimen, the resulting solution was centrifuged at 15,000 rpm for 3 min and then used for PCR.

In adults and pupae, the PCR success rates using species-specific primers and DNA barcode region were > 90% (Table 1). In all sequenced samples, the 658 bp sequences of DNA barcode region were identical to those previously obtained for *B. odoriphaga* using the destructive DNA extraction method. After nondestructive DNA extraction, all adult specimens could be accurately identified as *B. odoriphaga* based on the morphological characters observed from the permanent slides (Fig. 3). Therefore, this method could be applied for both the molecular and morphological identification of the adults of this species. When unknown species are newly collected during monitoring in Welsh onion fields, these species can be accurately identified using this method. However, in larvae and eggs, the success rates of PCR analysis using species-specific primers were not high (Table 1). Therefore, species identification of immature stages must be conducted using a destructive DNA extraction method.

Table 1. Success rate (%) of PCR using the nondestructive DNA extraction method for identification of *Bradysia odoriphaga*

Developmental Stage	*N*	PCR using species-specific primers of *Bradysia odoriphaga*	PCR of the DNA barcode region
Male adult	100	92	99
Female adult	100	100	100
Male pupa	100	93	98
Female pupa	100	98	100
Larva	100	51	85
Egg	100	13	25

(Modified from Arimoto et al. 2020a)

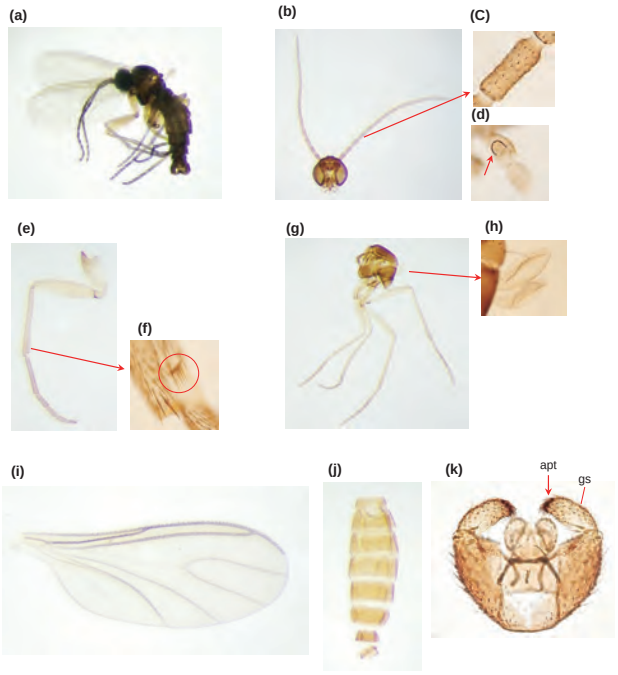

Fig. 3. Morphological characters of an adult male *Bradysia odoriphaga* after nondestructive DNA extraction
(a) Whole body; (b) head; (c) fourth flagellomere; (d) sensory pit of maxillary palpus; (e) right fore leg; (f) comb-like row of setae on inner side of fore tibia; (g) thorax, mid legs, and hind legs; (h) halters; (i) right wing; (j) abdomen, excluding hypopygium, ventral view; (k) hypopygium, ventral view (apt: apical tooth, gs: gonostylus)
The important characters for species identification are the ratio of length and width of the fourth flagellomere (c), color of halters (h), and position of apical tooth on gonostylus (k).
(Modified from Arimoto et al. 2020a)

Molecular detection of *B. odoriphaga* in bulk samples of sciarid flies

When *B. odoriphaga* first appears in a new area, it is considered that the number of individuals is very small. In such situations, it is necessary to detect one individual of this species in bulk samples of sciarid flies collected in the field. Therefore, molecular detection in bulk samples using species-specific primers was examined (Arimoto et al. 2020b).

For larvae and male adults, bulk samples (10, 100, 200, 400, and 800 individuals) composed entirely of *B. impatiens* individuals, except for one *B. odoriphaga* individual, were prepared. DNA was extracted from each sample using a destructive method and then used for PCR.

In each ratio, two bands were obtained after PCR using species-specific primers for *B. odoriphaga* (Fig. 4a, b). The sizes of the two bands were 709 and 324 bp, which corresponded to the previously reported sizes of *B. impatiens* and *B. odoriphaga*, respectively (Fig. 2).

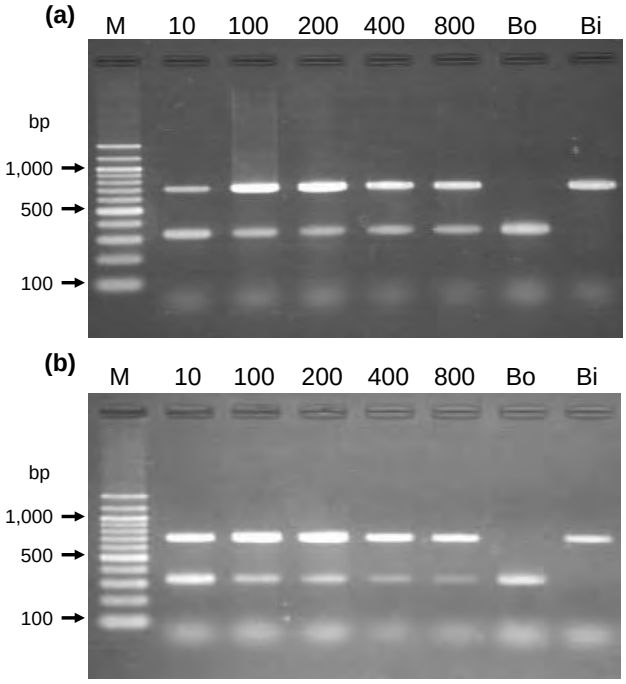

Fig. 4. Photographs of PCR-amplified products from one *Bradysia odoriphaga* individual in bulk samples of *Bradysia impatiens*
(a) Larvae; (b) male adults, M: 100-bp ladder DNA size marker; 10, 100, 200, 400, and 800: one individual of *B. odoriphaga* and remaining individuals of *B. impatiens* (Bo: one individual of *B. odoriphaga*; Bi: one individual of *B. impatiens*)
(Modified from Arimoto et al. 2020b)

These results indicate that *B. odoriphaga* could be detected in bulk samples of *B. impatiens* in any ratio examined by this method. In the monitoring of *B. odoriphaga* larvae, the presence of its larvae is checked by dipping damaged host plants in water (National Agriculture and Food Research Organization [NARO] 2020). Thus, this molecular detection method is applicable for the rapid detection of *B. odoriphaga*.

Relationship between the exposure period of *B. odoriphaga* adults caught on field traps and PCR success rates

The adults of *B. odoriphaga* are usually monitored in fields using yellow sticky traps (National Agriculture and Food Research Organization 2020). However, if the traps are left in the fields for a long time, the DNA of the captured adults may degrade, and PCR amplification may fail. It is necessary to know the duration of specimen exposure in fields that is acceptable to detect this species using PCR (Arimoto et al. 2020c).

One hundred and twenty adult specimens were manually adhered to each yellow sticky trap. Two traps were placed in an open area in direct sunlight, and two traps were placed in the shade of a building (Fig. 5). Traps were set during spring (May-July), summer (July-September), autumn (September-November), and winter (November-January) seasons, and each period lasted for 64 days. Sixteen specimens were collected from each trap on day 1, 8, 22, 36, 50, and 64 after initial trap placement. DNA was extracted from each specimen using a destructive extraction method and then used for PCR analysis with species-specific primers.

For all four study periods, the PCR success rate of

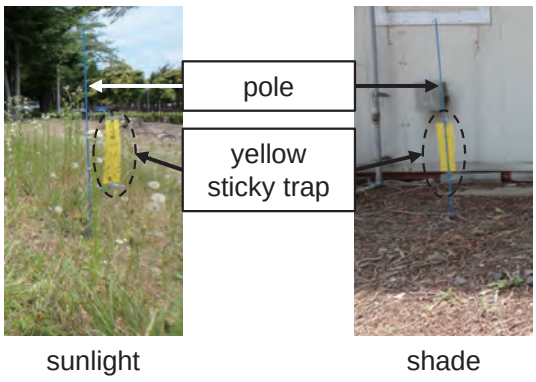

Fig. 5. Yellow sticky traps placed in study site
Only one of the two traps is shown for both sunlight and shade.
(Modified from Arimoto et al. 2020c)

sciarid specimens on traps tended to decrease as exposure period increased (Fig. 6). In almost all situations, the PCR success rates of specimens in direct sunlight were lower than those in the shade. These results may be due to DNA damage caused by UV radiation (Buck & Callaghan 1999). Even in the shade in summer, the PCR success rate was very low after 50 and 64 days of exposure. These results may be due to exposure to rainfall because DNA is hydrolyzed (Lindahl 1993). In this study, the PCR success rate after 8 days was > 80%, except for under direct sunlight during spring. In field monitoring, traps should be collected within 8 days, regardless of the season.

Conclusion

At present, the distribution of *B. odoriphaga* is limited to China, which is assumed to be the place of origin of this species, except for the Saitama and Gunma Prefectures in Japan (Sueyoshi & Yoshimatsu 2019). In recent years, the import volume of fresh vegetables, including Welsh onion, from China to Japan has increased (Fukase 2016). Therefore, it is necessary to pay attention to the invasion of *B. odoriphaga* into Japan. Likewise, the New Zealand government has also been concerned about the invasion of *B. odoriphaga* via fresh onion imported from China (Ministry for Primary Industries 2016).

Ishihara & Tagami (2020) examined the flight and walking durations of female and male adults of *B. odoriphaga* at five specific temperatures. They concluded that the dispersal ability of this species is not high and that the timing of dispersal is limited because of the low flight ability and reduced mobility at low temperatures in females. Therefore, in Japan, the expansion of *B. odoriphaga* may occur through domestic and international transportation of Welsh onion infested by the larvae of this species.

Welsh onion and carrot were heavily damaged when *B. odoriphaga* occurred in new areas in Japan (Omata 2017). In Saitama Prefecture, to prevent the expansion of this species, the presence of its larvae in Welsh onion has been checked by dipping damaged plants in water before shipment (Omata et al. 2019). In addition, it is important to conduct field monitoring using yellow sticky traps, followed by the rapid identification of collected specimens. Molecular detection in bulk samples may be useful for the efficient monitoring of this species.

In recent years, damage to Welsh onion by another sciarid fly, *P. scabiei*, was reported from Mie Prefecture, Japan (Plant Protection Office of Mie Prefecture 2019). PCR using species-specific primers for *B. odoriphaga* was conducted, and it was confirmed that the specimens were not *B. odoriphaga*. Therefore, this method was also useful for the rapid confirmation of whether sciarid specimens collected in the fields are *B. odoriphaga* or not in the prefectures where this species was not previously recorded. In this case, the specimens were finally identified as *P. scabiei* by DNA barcoding.

As described above, the molecular identification methods described in this article are highly practical. Thus, these may be applicable for the rapid identification of this species from specimens collected during the quarantine of imported plants.

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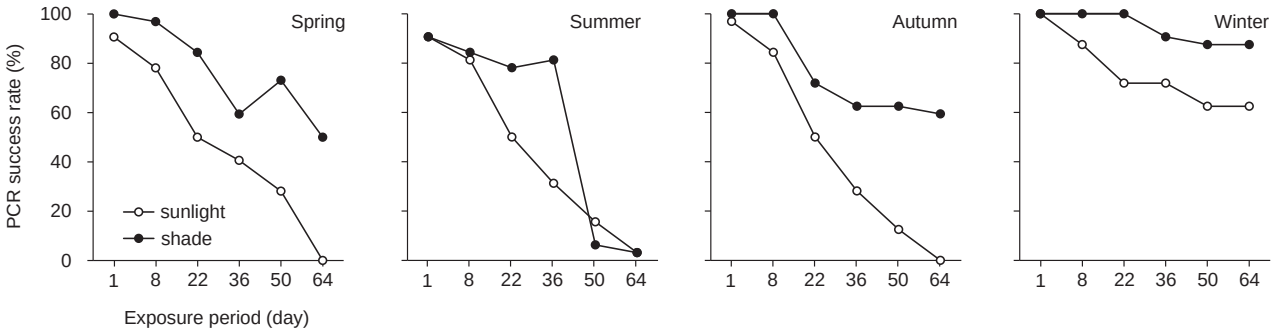

Fig. 6. Relationship between the exposure period of adult *Bradysia odoriphaga* on yellow sticky traps and PCR success rates
In the shade during spring, 26 specimens were used after 50 days, and four specimens were used after 64 days because some specimens fell off the yellow sticky traps.

(Modified from Arimoto et al. 2020c)

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