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Annual Occurrence Patterns and Three Dimensional Distribution of Mushroom Flies in *Pleurotus ostreatus* Cultivation Farms

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느타리버섯 재배에서 버섯파리의 연중발생패턴과 입체적 분포

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ABSTRACT: Among flies inside mushroom growing rooms in three farms, *Lycoriella ingenua* and *Megaselia tamilnaduensis* were the most common and dominant species during *Pleurotus ostreatus* cultivation in Korea. In the past, generally, during the incubation period, a low density of mushroom flies was observed in all farms. After the first harvest, mushroom flies density tended to increase sharply. However, many mushroom flies were observed in the summer, despite that season corresponds to the incubation period. This is because annual cultivation systems provide a safe overwintering place compared to seasonal selective ones. The ecology of mushroom flies varies greatly according to the cultivation system. We confirmed that a fallow period reduced the density of mushroom flies. From a survey of the three dimensional distribution of mushroom flies in a growing room, we observed that *M. tamilnaduensis* showed more positive phototaxis and a higher variation per point of capture than that by *L. ingenua.* Through this study, two mushroom fly species were identified in the survey farms, with markedly different three dimensional distribution patterns.

Key words: *Lycoriella ingenua*, *Megaselia tamilnaduensis*, *Pleurotus ostreatus*, Phototaxis

초 록: 최근 느타리버섯은 연중재배가 보편화되고 있으며, 재배량도 급격히 증가하고 있는 추세이다. 경북지역 느타리버섯 재배사에서 3년에 걸쳐 버섯파리 발생밀도를 조사한 결과 긴수염버섯파리와 버섯벼룩파리가 가장 우점하여 발생하였다. 일반적으로 균사배양기간에는 버섯파리 밀도가 모든 농가에서 낮았지만 1주기 수확후에는 급격히 증가하는 경향을 보였다. 하지만, 여름철에는 배양기간임에도 불구하고 많은 버섯파리가 관찰되었다. 이 현상은 연중재배체계의 보편화로 인해 계절적 선택재배와 달리 버섯파리가 재배사에서 안전하게 월동할 수 있는 서식처를 제공받았기 때문이다. 버섯파리의 생태는 버섯재배환경에 따라 달라질 수 있다. 계절적 선택재배농가와 연중재배농가의 버섯파리 발생패턴 차이를 통해 휴경이 버섯파리 발생밀도 감소에 중요한 역할을 한다는 것을 확인하였다. 버섯재배사 내부의 버섯파리 입체발생분포 조사를 통해 긴수염버섯파리보다 버섯벼룩파리가 빛에 더 민감한 종임을 확인하였다. 또한 2종의 입체적 발생양상은 매우 차이가 있는 것을 확인하였다.

검색어: 긴수염버섯파리, 버섯벼룩파리, 느타리버섯, 주광성

The Oyster mushroom, *Pleurotus ostreatus,* is the most frequently cultivated of numerous edible mushrooms in Korea (MAFRA, 2014), being also produced and consumed in China, America, Australia, and Europe (World Agriculture, 2015). Throughout the world, mushroom flies (Diptera) cause great damage and economic losses during mushroom cultivation (Wyatt, 1963; Clift, 1979; White, 1985; Kim and Hwang, 1996; Erler et al., 2011). The families Sciaridae and Phoridae are reported to produce the most severe damage. Among the Sciaridae, *Lycoriella ingenua* (syn. *L. mali* Fitch, *L. solani* Winnertz) has been reported to cause the most serious damages

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to mushroom production (Menzel and Mohrig, 1997; White et al., 2000, Menzel and Mohrig, 2000; Sinclair and Dorchin, 2010). *L. ingenua* was first recorded in Korea in 1999, also occurring in cultivations of *Lentinula edodes* and *Agaricus bisporus.* The occurrence of *L. ingenua* reduced yields of *Agaricus bisporus* crops by 17% (Cantelo, 1979).

Among the Phoridae family, *Megaselia halterata* is the most common mushroom pest. Although direct damage to the mushroom by *M. halterata* is not very important (Rinker and Snetsinger, 1984), the presence of Phoridae must be avoided, because they could act as vectors of the dry mold *Verticillium fungicola* (White, 1981). In Korea, *Megaselia tamilnaduensis,* which is the same species occurring in India (Mohan et al., 1996), was first reported in 2001 (Lee et al., 2001).

In this study, we identified these two dominant mushroom flies, the trends in their annual occurrence, and the spatial distribution patterns in mushrooms growing rooms for the development of an integrated management system of mushroom pest flies that cause damages to oyster mushroom crops.

Materials and methods

Morphological and molecular identification of mushroom flies

Adult mushroom flies were collected from *Pleurotus ostreatus* farms using a yellow sticky trap (25×15 cm) and an insect sucking device. Collected individuals were kept in vials with 75% alcohol. For identification and description, adult flies were mounted on microscope slides in Canada balsam. Before mounting, the head, wings, and male genitalia were dissected, dehydrated successively in 99% alcohol, and soaked in clove oil. Then, each specimen was placed into 1 or 2 drops of mounting media on a glass slide. Photographs were taken with a digital camera attached to a microscope (SMZ1500, Nikon) and images were combined using IMT iSolution Lite version 8.3 (IMT i-Solution Inc.). Sample preparation for scanning electron microscopy (SEM) was followed as referenced by Lee and Ahn (2015). A 10 nm coating of gold-palladium was applied on specimens using a sputter coater. Specimens were then observed on a LEO1450VP SEM (Zeiss) with a 20 kV voltage and magnifications ranging between 20× and 11,000×.

Molecular identification was performed using the DNA barcoding method, which has recently been used for the identification of common insect species. In this study, mitochondrial cytochrome C oxidase subunit I (COI) gene, which is part of the typical insect DNA barcoding genes use for taxonomic identification, like nuclear 18S rRNA gene and 16S mDNA and rDNA, was used for DNA barcoding analysis. Total genomic DNA was extracted from somatic tissue of individuals using a DNeasy® Blood and Tissue kit (QIAGEN, Inc.). PCR amplification was done using forward primer (LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′ and reverse primer HCO2198 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′; Folmer et al., 1994). PCR was carried out using the Applied Biosystem (Gene Amp PCR System 2700) with 50 µl reaction volumes, containing 2 µl of (2.5 mM) forward primer 2 µl of (2.5 mM) reverse primer, dNTPs (10 mM,) 10× Ex Taq Buffer (20 mM Mg^{2+}), TaKaRa Ex Taq polymerase (5 U/µl), and 3 µl of genomic DNA template. Thermocycling consisted of 35 cycles of initial denaturation at 94℃ for 3 min, denaturation at 94℃ for 30 s, annealing at 50℃ for 30 s, and extension at 72℃ for 1 min, followed by a final extension at 72℃ for 10 min. PCR products were clean using a QIAquick PCR purification kit (QIAGEN, Inc.) and directly sequenced at Solgent Inc. (Daejeon, Republic of Korea). Sequences were aligned using the ClustalX method (version 2.0.11). Phylogenetic analysis was performed with MEGA version 6.0 using the maximum likelihood method, and sequence distance was calculated with 1,000 replication of the bootstrap value.

Annual occurrence patterns in mushroom farms

To assess the annual occurrence patterns of mushroom flies, a survey was conducted in Goryeong-gun (Farm A), Chilgok-gun (Farm B), and Gumi-si (Farm C), because those cities have more than 10 oyster mushroom farms. Farm A and B have annual cultivation system, but farm C has a seasonal selective cultivation system. Surveyed farms' mushroom cultivation activities dated from between 5 to 30 years. Because there is little difference in starting time, facilities, and cultivar, comparing average values among the three-points was difficult. Consequently,

results were analyzed for each investigated point. All farms in the three cities were surveyed for a period of three years in a two-week interval. From March 2013 to October 2015, we placed 10 yellow sticky traps (25×15 cm) in each farm and the traps were changed every two weeks after installation. Fly species composition and number of flies were assessed. Major damage patterns were photographed.

3D distribution of mushroom flies in cultivation room

To estimate the 3D distribution of mushroom flies, a survey was performed at the farm A in Goryeong-gun. The survey was carried out from October 2013 to October 2015. Forty yellow sticky traps (25×15 cm) were installed inside the mushroom growing room to investigate fly dynamics. For more detailed investigation, excluding the ceiling areas which are 1 to 4 points, the 36 points were classified into pathway which has from 14 to 31 points and surface area which has from 5 to 13 points and from 32 to 40 points. According to the distance from entrance, we divided entrance which has 14, 17, 20, 23, 26, and 29 point, 7 m from entrance which has 15, 18, 21, 24, 27, and 30 point, and 14 m from entrance which has 16, 19, 22, 25, 28, and 31. The house is 7.0×15.0×4.5 m (width × length × height) with two cultivation shelves (1.5×12.0×0.55 m, width × length × height) inside (Fig. 1). Distance between trap was 4 m in before and behind interval, 1.5 m in horizontal spacing, and 0.55 m in height. Traps were changed every two weeks during the study. Fly species composition, number of flies, and temperature at each point were investigated. Position of traps was as shown in Fig. 1.

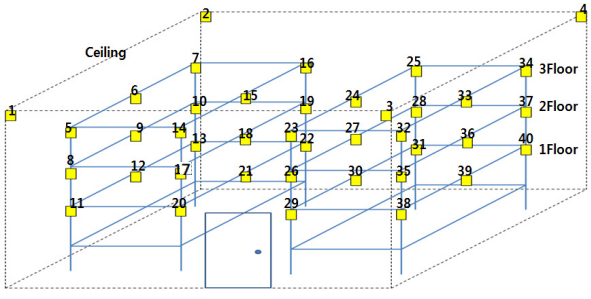

Fig. 1. Diagram of yellow sticky trap position to assess three dimensional distribution pattern of mushroom fly species *Lycoriella ingenua* and *Megaselia halterata*.

Results and Discussion

Morphological and molecular identification of mushroom flies

L. ingenua and *M. tamilnaduensis* were the most common and dominant species in the oyster mushroom cultivation house. *L. ingenua* and *M. tamilnaduensis* were easily distinguished when captured in the traps because of their size differences (Fig. 2). Morphological and ecological characteristics of *L. ingenua* were already reported by Shin et al. (2012) and Lewandowski et al. (2004). *L. ingenua* is easily recognized by the conspicuous bristles on the basal lobe of male genitalia (Fig. 3). Sequences of the mitochondrial COI showed a 100% match with *L. ingenua* (JN378575, GenBank; Fig. 4). To identify *M. tamilnaduensis*, some samples were observed (Fig. 5). It was confirmed that the species is the same as that reported by Mohan et al. (1996) and Lee et al. (2001). Compared to Indian specimens, our specimens have slightly dusky palps and differences in the dimensions of the sixth abdominal tergite (Fig. 6). According to the sequence analysis of the COI region of *M. tamilnaduensis*, our sample was the most similar to *Megaselia* sp. (KM637465.1, GenBank; Fig. 4). However, genetic information on the identified *M. tamilnaduensis* COI was not registered. We registered the COI sequence information of *M. tamilnaduensis* with the number KX774369 in NCBI GenBank, as biological information.

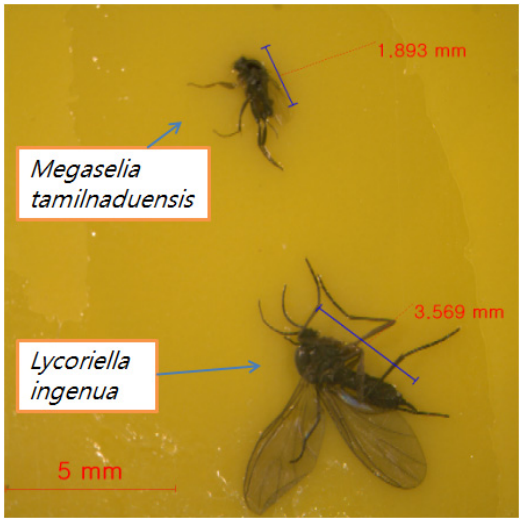

Fig. 2. Body length comparison in two major mushroom flies.

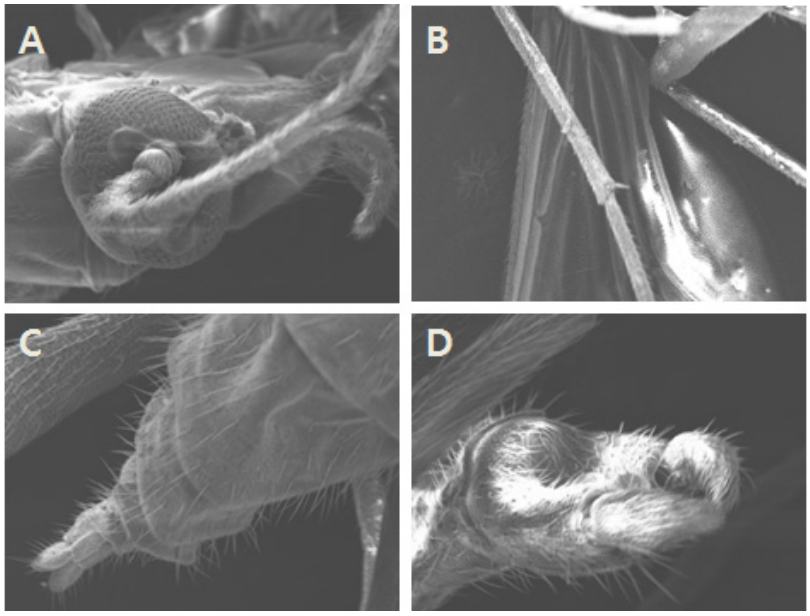

Fig. 3. Scanning electron microscope (SEM) photographs of *Lycoriella ingenua* morphological characteristics. (A) Head parts, (B) fore tibia bristles, (C) end of abdomen in a female, (D) end of abdomen, male genitalia.

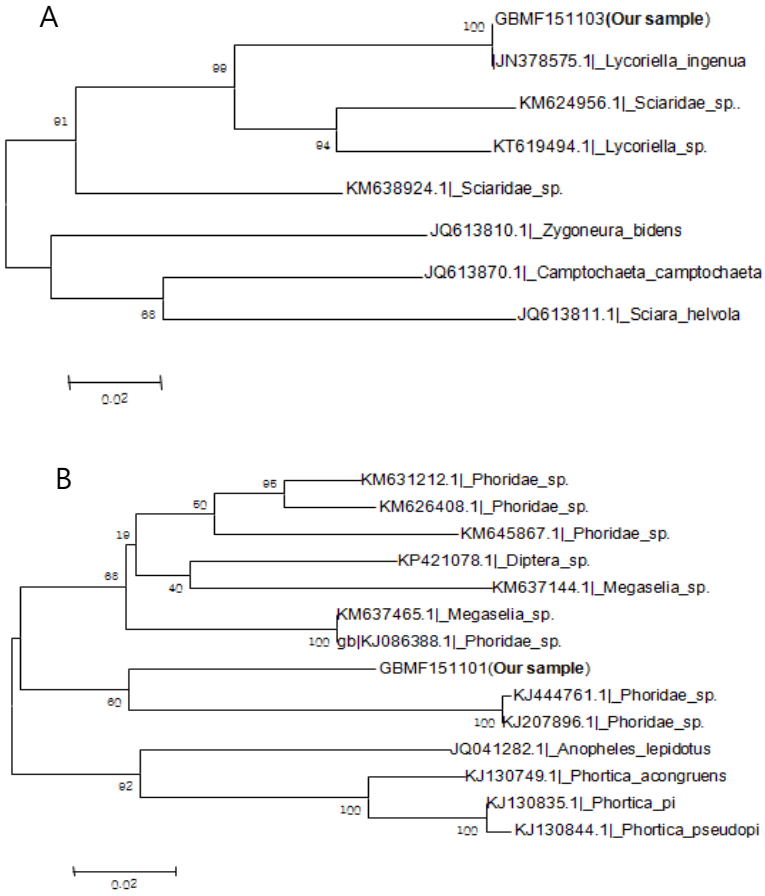

Fig. 4. Phylogenetic tree based on COI sequences of the mushroom flies *Lycoriella ingenua* (A) and *Megaselia tamilnaduensis* (B). Maximum likelihood tree inferred.

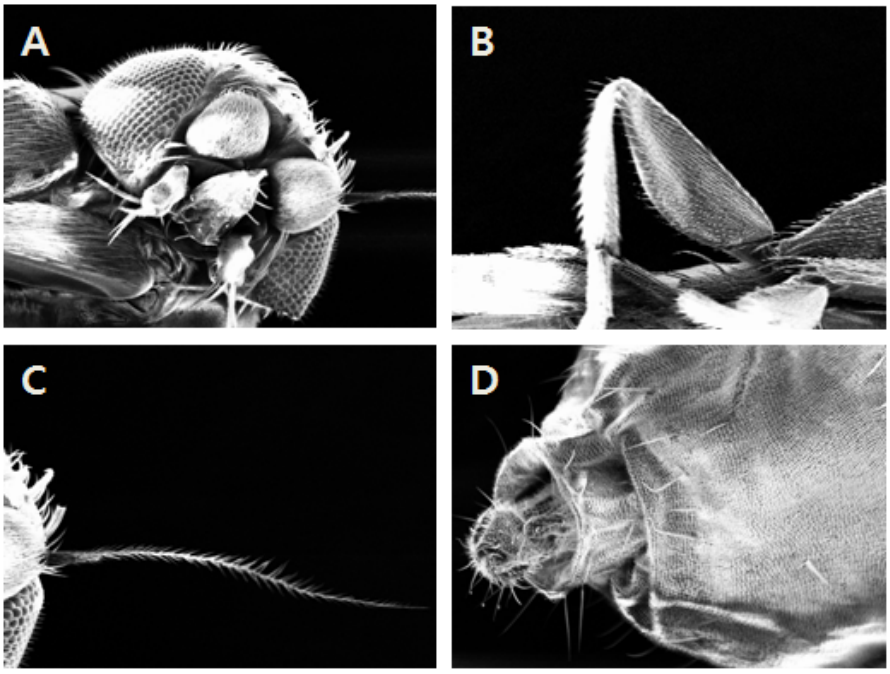

Fig. 5. Scanning electron microscope (SEM) photographs of *Megaselia tamilnaduensis* morphological characteristics. (A) Compound eye and mouth parts, (B) leg femur, (C) antenna, (D) abdominal parts.

Fig. 6. Morphological characteristic of *Megaselia tamilnaduensis.* (A) Female frons with bristles, Korean sample, (B) female abdominal tergites, Indian sample, (C) female abdominal tergites, Korean sample.

Annual occurrence patterns in mushroom farms

From 2013 to 2015, the annual occurrence patterns of *L. ingenua* and *M.tamilnaduensis* were as follow (Fig. 7). During the incubation period, mushroom fly density was low, tending to increase sharply after the first harvest, in all farms. Lee et al. (1999) reported that mushroom fly occurred 30 days after spawning. However, during the spawning and incubation periods in summer, a high density of flies was observed in two farms. In the spring, *L. ingenua* was dominant, being replaced in dominance by *M. tamilnaduensis* from summer to autumn, producing most damage to the mushroom. From December, only *L. ingenua* was observed, but the number of flies was very low. The period of the mushroom fly occurrence in 2014 was longer than that in 2013, because the average temperature of early winter in 2014 was higher than the previous year showing also a warmer autumn (Fig 7A, B). In particular, *L. ingenua* succeeded overwintering in the growing room during the sixth cultivation period (Fig. 7B). As a result, the density of *L. ingenua* was higher than that from late February, 2014. In addition, March density of *L. ingenua* was the highest of the three years period. Clift and Toffolon (1981) studied the biology and population dynamics of *L. auripila* in the Windsor area, west of Sydney. They indicate that in Sydney, winters are not colder than that in England and with Pennsylvania in the United States to restrict the activity of flies. On the latter two locations, winters are too cold to allow the re-infestation of mushroom farms from surrounding areas. Similar to Australia, Korea will become warmer in winter, and thus, this phenomenon will be more frequent.

The overall pattern of occurrence at investigation farm C differed from other survey points. The occurrence of *M. tamilnaduensis* was delayed about one month when compared to the other farms. This could be the result of the fallow during winter, which may have delayed the occurrence of flies. After a one-month fallow, there was a low density of mushroom flies in the second cultivation. The density of *L. ingenua* increased from early March in the third cultivation. The highest density of mushroom flies was more than double that of other farms because of the experience in mushroom flies management. However, the density of flies was low in the fourth cultivation period after a fallow of two months.

In 2015, for control of mushroom fly, survey point A and B were carried out after taking a two or more months break emptying all cultivation rooms in order to extinguish flies. As a result, density of mushroom flies lowered in two farms. We could confirm that fallows have a great effect on density reduction of mushroom flies of next cultivation.

From this result, the ecology of mushroom flies could be greatly changed because annual cultivation systems are able to provide a safe overwintering place, compared to seasonal selective cultivation systems. Therefore, the development of appropriate control systems is necessary to prevent damage from mushroom flies because annual cultivation systems are common in farms. Records provide crucial information on the effectiveness of a farms' hygiene program (Shamshad, 2010). Our experiments with *L. ingenua* and *M. tamilnaduensis* contribute to the knowledge of the phenology of these species.

Survey of 3D distribution of mushroom fly in cultivation room

Previous studies have examined horizontal distribution of mushroom fly (Lee et al., 1999). This is the first report of the three dimensional (3D) distribution of mushroom flies at an oyster mushroom growing room in Korea. In this study, we investigated the density of mushroom fly within a three dimensional space at 40 points from the room entrance. From October 2013 to October 2015, 581,383 *L. ingenua* flies were captured in yellow sticky traps (Fig. 8A). The average number of captured flies per one investigation point was 14,534 ± 3,312 flies. Differences of *L. ingenua* distribution according to the height have not been confirmed. In addition, there was only 4% difference in the distribution of *L. ingenua* between the pathway and the surface area. There were only differences according to the distance from the entrance, with 144,197 flies at the entrance, 171,485 flies 7 m from the entrance, and 205,914 flies 14 m from the entrance (closed door). The further from the entrance, the higher was the density of *L. ingenua*.

From October 2013 to October 2015, 901,399 *M. tamilnaduensis* flies were captured in yellow sticky trap (Fig. 8B). The average number of captured flies per investigation point was 22,535 ± 10,335 flies. Density of *M. tamilnaduensis* at the third floor was 39% and 28% higher than at the first and second floor,

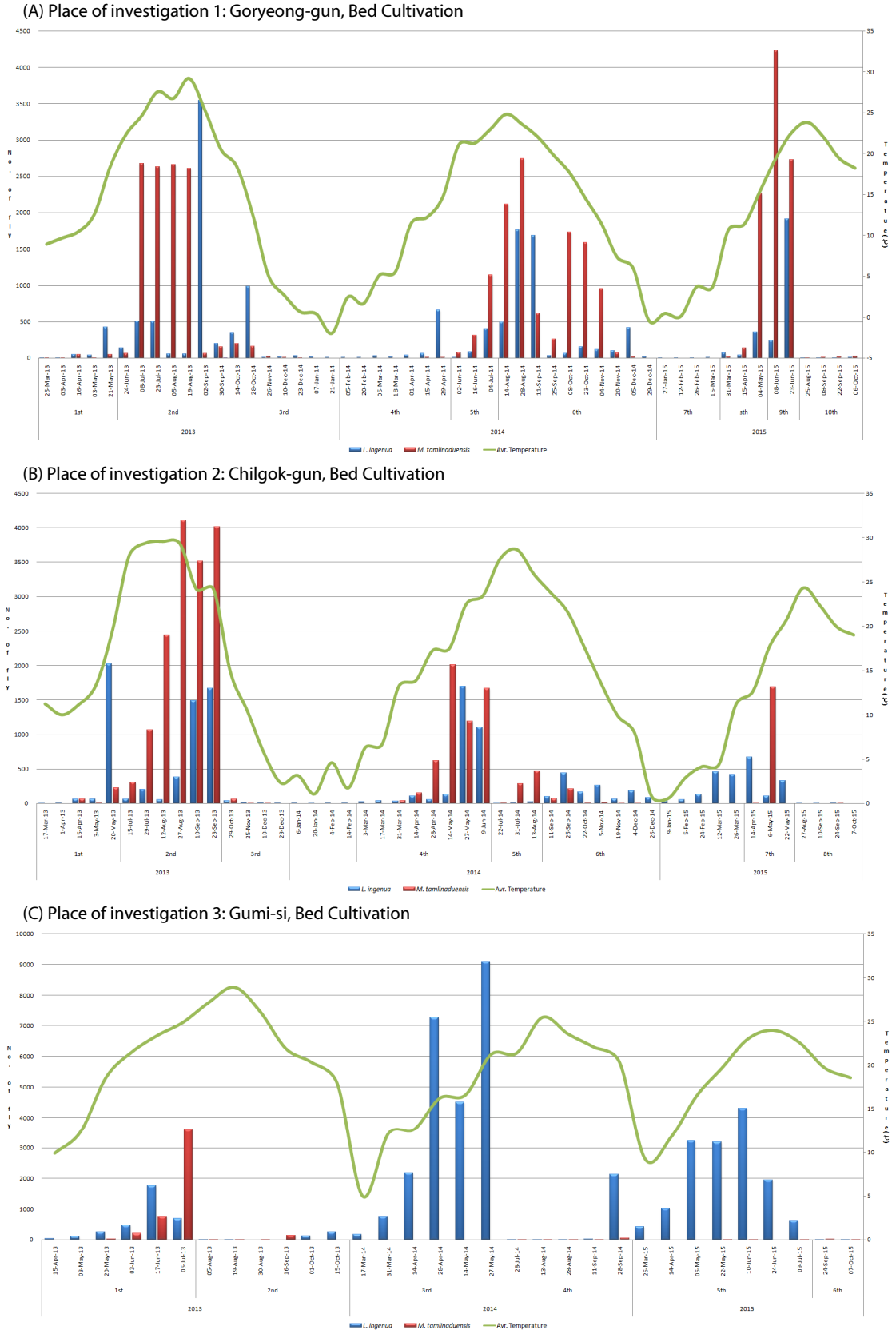

Fig. 7. Occurrence pattern of mushroom flies between 2013 and 2015 by mushroom farm (A: Goryeong; B: Chilgok; C: Gumi).

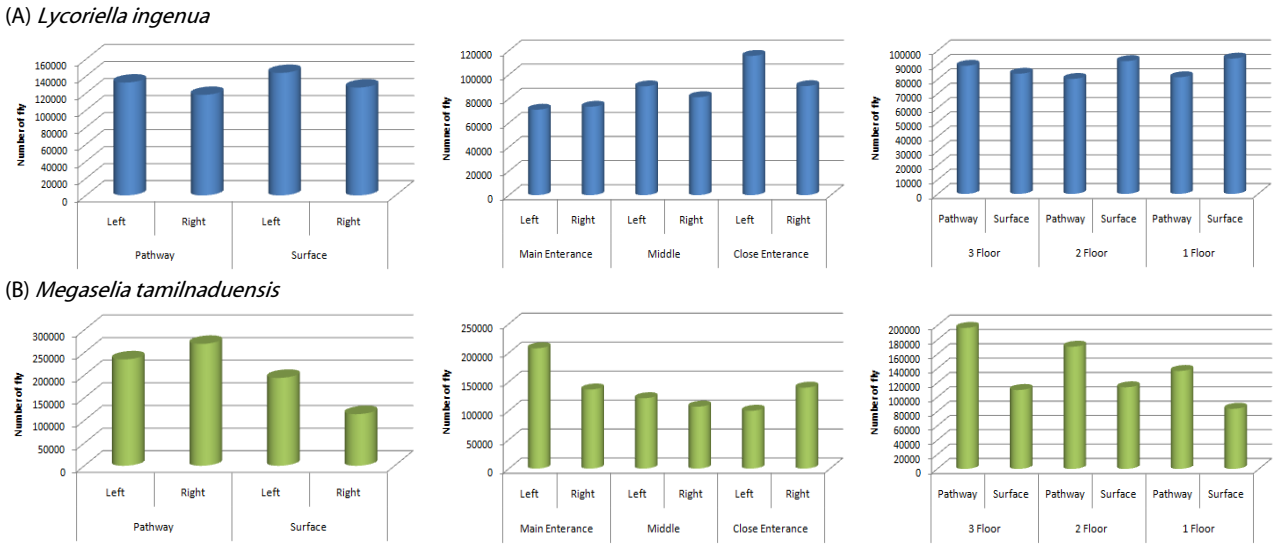

Fig. 8. *Lycoriella ingenua* and *Megaselia tamilnaduensis* density inside mushroom growing rooms.

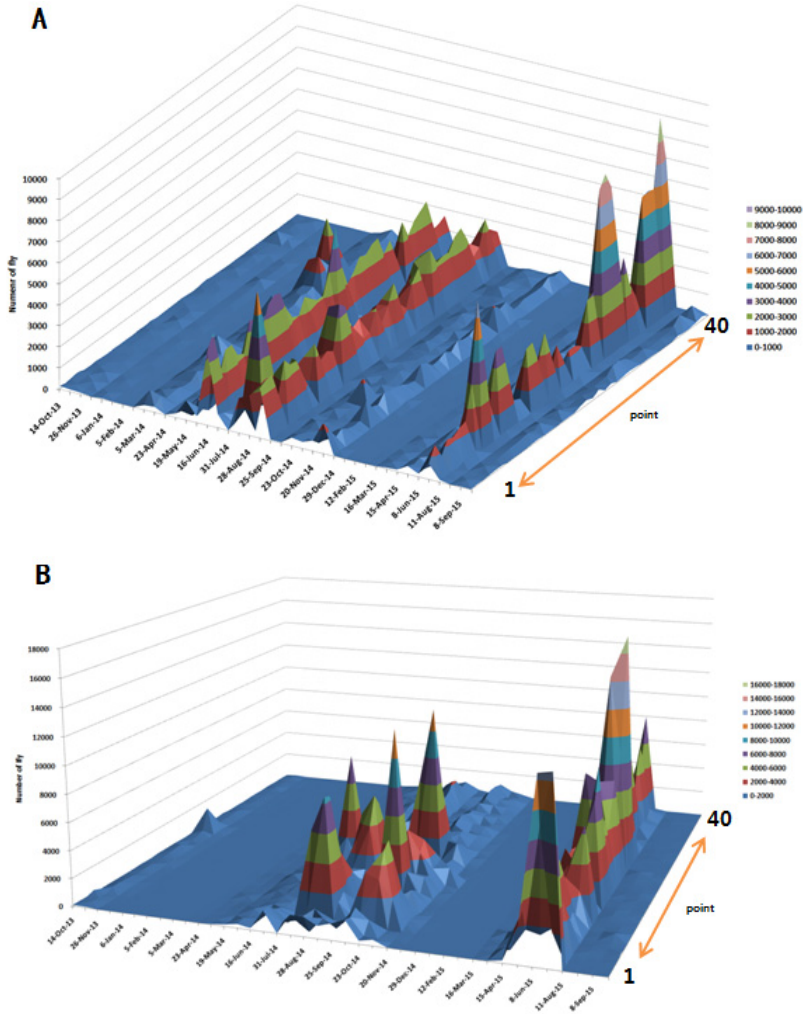

Fig. 9. Annual three-dimensional spatial distribution of *Lycoriella ingenua* (A) *and Megaselia tamilnaduensis* (B) inside growing room. Assessed in 40 points between 2013 and 2015.

respectively. There was a notable difference from the distribution of *L. ingenua.* Around pathway density was 63% higher than *L. ingenua* between pathway and surface points density. Unlike *L. ingenua*, the density of *M. tamilnaduensis* was higher near the entrance than in areas further distant. At the entrance, 344,439 flies were trapped, whilst at 7 m and 14 m (closed door) from the entrance 228,467 and 239,536 flies were trapped. The entrance side of the growing room had a large influx of light, which attracted *M. tamilnaduensis*. Girard et al. (1972) said that anyone who regularly visit mushroom farms is astonished by the accumulation of dead phorid adult about the doors, beneath the windows and near cracks where light enters. We confirmed the same phenomenon. When comparing the two species of mushroom files inside the mushroom growing room, 320,016 more flies of *M. tamilnaduensis* than *L. ingenua* were captured in the same period. Variation per point of captured was bigger for *M. tamilnaduensis* than for *L. ingenua.* Through this research, two species of mushroom flies with a markedly different three dimensional distribution pattern, confirming that *M. taminaduensis* has a higher positive phototaxis compared to *L. ingenua*. Such behavior could be used as a basis for mushroom flies control in oyster mushroom cultivation. In our survey, the occurrence of mushroom flies in the investigation points changed through the mushroom cultivation seasons (Fig. 9).

Control of mushroom flies has focused on insecticides, and the pests have developed resistance against the most frequently used chemicals. Globally, the UK has the most significant resistance problems (Steve et al., 2000). Difficulties in controlling mushroom flies with insecticide have stimulated a renewed interest in alternative methods (Hussey, 1972; Hussey et al., 1969). This result will be used as the basis for the development of an alternative mushroom fly control system for annual cultivation systems, which has been used in recent years.

Acknowledgments

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