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Short Communication

Determination of coleopteran insects associated with spore dispersal of *Cryptoporus volvatus* (Polyporaceae: Basidiomycota) in Korea

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ABSTRACT

The veiled polypore, *Cryptoporus volvatus*, is distributed widely in North America and East Asia and is believed to have a mutualistic relationship with coleopteran species—the fungus providing food and shelter in basidiocarps and beetle helping disperse spores.
Seventy fresh basidiocarps of *C. volvatus* were collected from the Japanese red pine (*Pinus densiflora*) in the spring season of 2013 from two sites in Korea. A total of 251 insects (101 adult and 150 larvae) were collected from the inside of basidiocarps and identified using morphology and mitochondrial cytochrome c oxidase subunit I (COI) sequences. Six species belonging to five coleopteran families were identified. The number of spores attached to the bodies of adult insects was counted and average spore counts for each of the six species ranged between 1.0×10^4 and 5.2×10^5 spores/individual. Across localities, three species were shared (*Aethina suturalis*, *Trogossita japonica* and *Parabolitophagus felix*) and carried spores at high densities on their bodies, making them more likely to aid in spore dispersal.

Introduction

Cryptoporus volvatus, a wood decay fungus widely distributed in North America and East Asia (Gilbertson and Ryvarden, 1986; Kadowaki, 2010a), is a polypore with unique basidiocarp morphology, having its pores enclosed by a subtended volva (Fig. 1). This special structure results in the majority of spores accumulating on the inner surface of the sheath (Ingold, 1953). It is believed that there is a mutualistic relationship between *C. volvatus* and a variety of coleopteran species, with the fungus providing food and shelter in the basidiocarp and beetle helping disperse spores (Hubbard, 1892; Zeller, 1915; Bassey, 1950; Ingold, 1953; Borden and McClaren, 1970; Setsuda, 1995; Alexopoulos et al., 1996; Kadowaki, 2010b).

The function of the unique basidiocarp has not been resolved. Harrington (1980) believes that the basidiocarp morphology is a xerophytic adaptation, while other researchers believe it is an adaptation for a fungus–insect mutualism. Some lines of evidence supporting a fungus–insect mutualism are (1) basidiocarps usually grow from bore holes of coniferous bark beetles (Setsuda, 1995), (2) many beetles live inside the basidiocarp (Borden and McClaren, 1970; Setsuda, 1995; Kadowaki, 2010b), and (3) *C. volvatus* has been cultured from the bodies of in-flight beetles (Castello et al., 1976). A goal of our study is to further evaluate the possibility of a mutualistic relationship between *C. volvatus* and associated coleopteran species.

Although the potential fungus–insect relationship of *C. volvatus* is regularly studied in North America (i.e. Castello et al., 1976; Harrington, 1980) and Japan (i.e. Setsuda, 1993, 1995; Kadowaki, 2010a,b), this topic has been poorly studied in Korea. In this study, we identify the coleopteran diversity associated with *C. volvatus* by using morphology and the mitochondrial cytochrome c oxidase subunit I gene (COI), as well as count the number of spores attached to the bodies of adult insects to evaluate their role as spore dispersers.

Materials and methods

Sample collection

Fresh basidiocarps were collected from the Japanese Red Pine (*Pinus densiflora*) during the spring season of 2013 from two sites in North Gyeongsang Province: Noeum Mountain, Sangju City (April 5) and Maebong Mountain, Yecheon County (April 3, May 6). Basidiocarps within arms reach were collected from 2–3 trees at each site. Basidiocarps were smaller and present in higher densities at Noeum compared to Maebong. A total of 70 basidiocarps were collected for this study: 30 from Noeum and 40 from Maebong.

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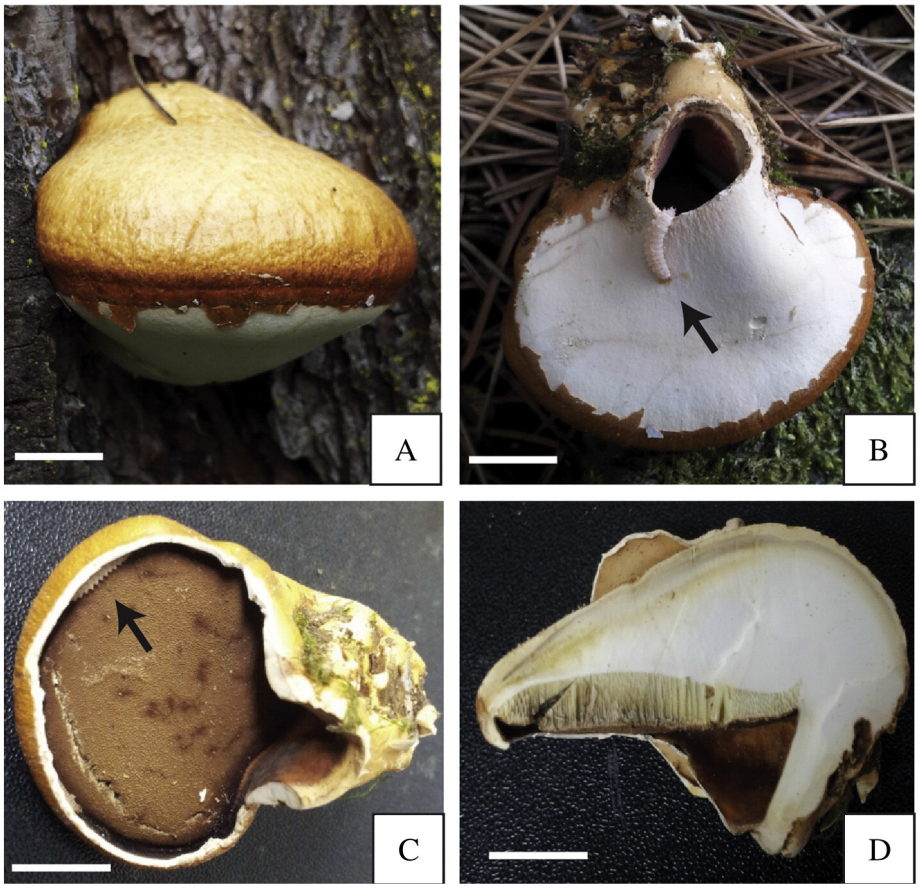

Fig. 1. Photographs of *Cryptoporus volvatus* basidocarp morphology. (A) basidiocarp growing attached to *Pinus densiflora*, (B) underside view, (C) basidiocarp with the subtended volva removed, exposing the pore surface, (D) cross sectional view. Arrows indicates the presence of a larval *Aethina suturalis* found associated with the basidiocarp. Scale bars represent 1 cm.

Morphological and molecular identification of coleopteran species

Adult and larval beetles were removed from the inside of basidiocarps and stored in vials containing 99% alcohol. Adults were stored separately while larvae from a single basidiocarp were stored in the same vial. Specimens were first grouped and identified based on morphology using species descriptions and identification keys (Jung, 2012; Jung and Park, 2013). Next, 1–4 specimens of each morphological group were selected for DNA sequencing. For small beetles, the entire specimen was used for extraction, while for larger beetles, a portion of the abdomen was dissected and used. DNA was extracted using a modified CTAB extraction protocol (Rogers and Bendich, 1994), with the addition of proteinase K. The universal primers for COI, LCO1490 and HCO2198 (Folmer et al., 1994), were used for DNA amplification. Polymerase chain reaction (PCR) was performed on a C1000™ thermal cycler (Bio-Rad, CA, USA) using the Maxime PCR PreMix-StarTaq (Intron Biotechnology Inc., Seoul, Korea) in a final volume of 20 μL containing 10 pmol of each primer and 1 μL of DNA. PCR conditions were 95 °C for 3 min, followed by 5 cycles of 95 °C for 1 min, 45 °C for 1 min, 72 °C for 1 min, 30 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 5 min. PCR products were electrophoresed through a 1% agarose gel stained with loading STAR (Dyne Bio, Seoul, Korea) and purified using the Expin™ PCR Purification Kit (GeneAll Biotechnology, Korea) according to the manufacturer's instructions. Sequencing was done in both forward and reverse directions using the PCR primers. The DNA sequencing was performed by Macrogen (Seoul, Korea), using an ABI3700 automated DNA sequencer.

Sequences were assembled, proofread, and aligned using Geneious v5.3.6 (Biomatters). Additional sequences from GenBank (http://www.ncbi.nlm.nih.gov/) were included in the analyses, both top hits from BLAST searches and, when available, COI sequences from taxa matching morphological identifications. Although standard COI barcoding primers were used (Folmer et al., 1994), many of the available sequences on GenBank did not overlap with this COI fragment. Thirty-six relevant reference sequences available on GenBank were downloaded and used in the preliminary molecular analysis for identification. For the final phylogenetic analysis, all distantly related taxa were removed, resulting in 16 new sequences and six reference sequences of closely related species from GenBank. Maximum likelihood (ML) phylogenetic analyses were run using RAxML v8.0.2 (Stamatakis, 2014) using the GTRGAMMA model of evolution for tree inference and 1000 bootstrap replicates. After species identification, intraspecific p-distances were calculated for all species with greater than one sequenced specimen. To compare community structure across sites, the Sorensen's similarity index (QS) was calculated (Sorensen, 1948).

Counting C. volvatus spores attached to the bodies of adult insects

For each of the 101 adult specimens, spore counts were repeated four times, then averaged. To calculate an average spore count for species with multiple specimens, an average was taken from all spore count trials. The methodology of counting spores is as follows. Prior to DNA work, each tube holding a single adult insect in 1 mL 99% ethanol was vortexed at 12,000 rpm for 10 s to dislodge spores from the surface of the insects. Next, insects were removed and placed in a new 1.5 mL tube with 99% ethanol for storage. Immediately before measurement, the first tube was vortexed briefly to evenly mix the suspension, and 5 μL of ethanol was placed on a hemocytometer for counting spores. The total spore count per trial was multiplied by 10^4 to calculate the

Table 1
Identification and instraspecific p-distances of coleopteran species found inside *Cryptoporus volvatus*. For the specimen count, the number inside the parentheses indicates the number of larval specimens.

Family	Species	Specimen count		No. sequenced	Max p-dist
		Maebong	Noeum		
Cucujidae	*Pediacus japonicus*	1	0	0	n/a
Mycetophagidae	*Mycetophagus attennatus*	1	0	1	n/a
Nitidulidae	*Aethina suturalis*	67 (66)	17 (84)	9	0.001
	Glischrochilus ipsoides	3	0	2	0.003
Tenebrionidae	*Parabolitophagus felix*	3	4	2	0.001
Trogossitidae	*Trogossita japonica*	1	4	2	0.014
	TOTAL	142	109		

number of spores per mL (i.e. the complete sample). Spore counts were performed with a light microscope (Nikon 80i) at 200× magnification. To estimate the spore coverage on each insect, the surface area of specimens was measured. The surface area of representative specimens for each species was estimated by measuring its length, width, and height. Next, the average spore coverage of each species was calculated by dividing the average spore count by the estimated surface area.

Results

A total of 251 coleopteran specimens (101 adult and 150 larvae) were collected. Six species (*Pediacus japonicus*, *Mycetophagus attennatus*, *Aethina suturalis*, *Glischrochilus ipsoides*, *Parabolitophagus felix*, *Trogossita japonica*) were identified from adult specimens based on morphological characters (Table 1). All larvae were of a single morphotype, but due to the lack of distinguishing morphological features, they could not be identified.

A total of 16 representative specimens of the different species (including larvae) were sequenced for the COI gene (GenBank Accession Numbers: KJ480776–77, 80–93). The single specimen identified as *P. japonicus* based on morphology was not successfully sequenced. Although the lack of reference data limited our ability to verify morphological identifications with COI data, sequence data helped us classify the unidentified larval specimens (Fig. 2), as they had identical or near identical sequences to adult *Aethina suturalis* (p-dist = 0–0.001). For the four species with more than one specimen sequenced, intraspecific p-distances were low, ranging between 0 and 0.014 (Table 1). The Sorensen's similarity index is a measure of community similarity, with QS = 1 being identical and QS = 0 being dissimilar. The QS score

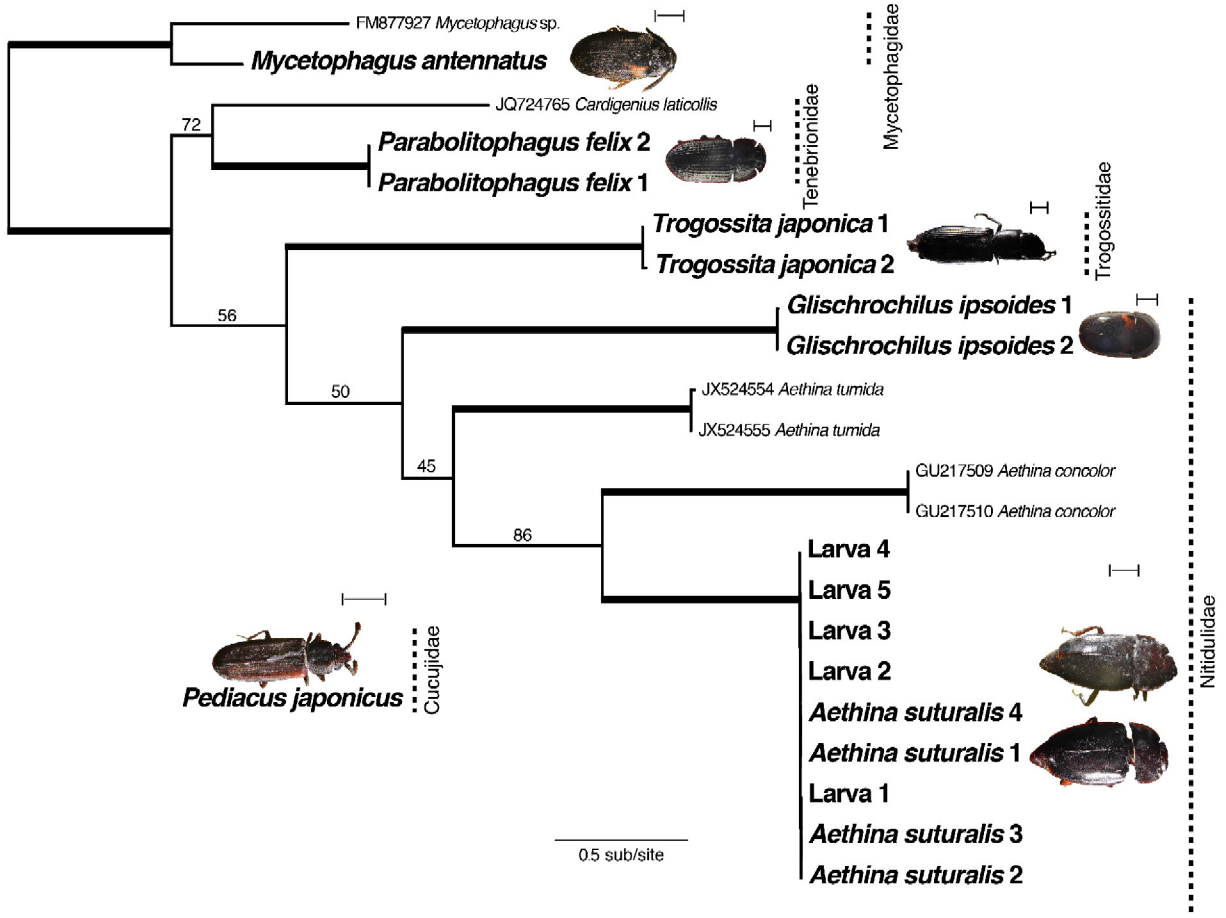

Fig. 2. Maximum likelihood tree of the mitochondrial COI gene. Taxa with photographs and names in larger, bold font are the species found inside *Cryptoporus volvatus*. Thicker branches in the phylogeny represent bootstrap values ≥90. Scale bars for each genus represent the size of the specimen photos (10 mm). *Pediacus japonicas* was not successfully sequenced, but was identified using morphology.

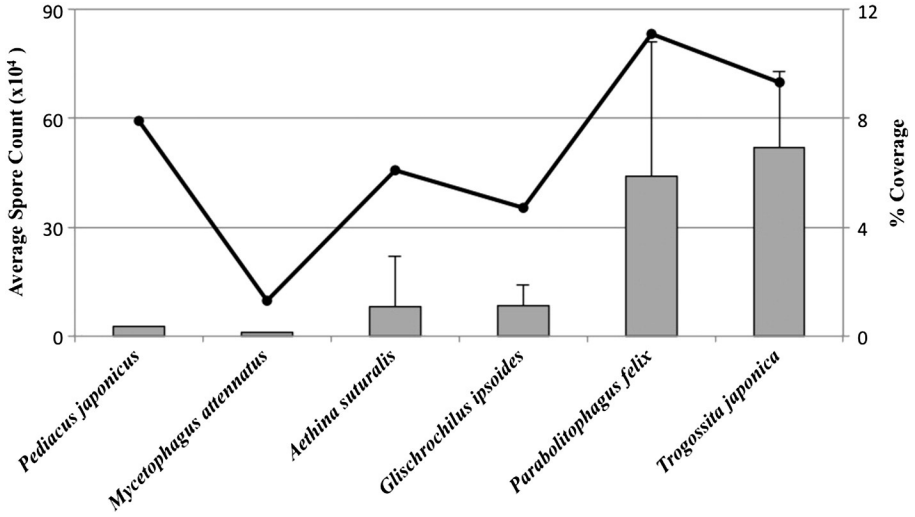

Fig. 3. Summary of average spore count (bar graph) and an estimate of the percent of the insect surface area covered by spores (line graph) of the six species identified in this study.

between Maebong and Noeum was relatively high (QS = 0.6) in relation to Korea–Japan comparisons (QS = 0.08–0.25).

All beetles found inside *C. volvatus* had a spore count ranging between 1.0×10^4 and 5.2×10^5 spores per species (Fig. 3). Spore count variation between individuals of the same species was high; of the seven *P. felix* specimens, average spore counts for individuals differed by almost 10^2 (6.8×10^4–1.1×10^6) (Fig. 3). Spore coverage accounted for approximately 5–10% of an individual's surface area (Fig. 3).

Discussion

A total of six coleopteran species (*Pediacus japonicus*, *M. attennatus*, *A. suturalis*, *G. ipsoides*, *Parabolitophagus felix*, *T. japonica*) were identified inside *C. volvatus*. Molecular data helped in classification of unidentified larval specimens, as they were identical to adult *A. suturalis*. Three beetle species (*A. suturalis*, *Parabolitophagus felix*, *T. japonica*) were shared across the two sites in our study. Based on Sorensen's similarity index, the coleopteran communities found in *C. volvatus* at Maebong and Noeum were relatively similar (QS = 0.6). In comparison to studies performed in Japan, insect diversity in Korean *C. volvatus* is much lower (Setsuda, 1993; Kadowaki, 2010b; Kadowaki and Yamazoe, 2011). Species richness at different sites in Japan ranged between 7 and 51 species, and often included other insect groups (Setsuda, 1993; Kadowaki, 2010b; Kadowaki and Yamazoe, 2011). QS scores comparing Korean and Japanese sites quantified this difference (QS = 0.08–0.25), but it is unclear whether this is a true difference due to geography or an artifact of different sampling efforts.

In our study, we also evaluated the possibility of coleopteran species being vectors of spore dispersal by quantifying the number of spores attached to their bodies. All species had spores attached to their bodies, with *Mycetophagus attennatus* having the lowest number (1.0×10^4), and *T. japonica* having the highest number (5.2×10^5) (Fig. 3). In general, insects with a larger surface area had higher spore counts. The next step in understanding whether fungal spores are dispersed is to understand the ecology and life history of these beetle species. We discuss the available data below.

The dominant species found in our study was *A. suturalis*, accounting for 93.7% and 68% of the adult beetles found in Maebong and Noeum, respectively, and 100% of larvae at both sites. There have been no ecological studies focusing on *A. suturalis*, but taxa in the same family (Nitidulidae) are known to feed on and disperse fungi (Appel et al., 1990; Cease and Juzwik, 2011; Jung and Lee, 2011; Jung and Park, 2013). The presence of both adult and larval *A. suturalis* leads us to believe that *A. suturalis* not only feeds on, but also breeds inside *C. volvatus*. These facts that *A. suturalis* is found in high densities inside *C. volvatus* and has a life history tied closely to *C. volvatus* make it a likely candidate of major spore dispersal.

Two identical species found inside *C. volvatus* in both Korea and Japan were *P. felix* and *T. japonica*. In Korea and Japan, *P. felix* can be found on several fungal hosts (*C. volvatus, Ganoderma lucidum, Cryptoderma pin, Fomitopsis* sp., and *Fomes* sp.) (Kim and Kim, 2002; Jung and Lee, 2011). In Japan, *P. felix* is known to both feed and breed inside the volval chamber of *C. volvatus*, being present in the basidiocarp in the spring and overwintering under the tree bark (Setsuda, 1995; Kadowaki, 2010b). *Trogossita japonica*, on the other hand, is a predator of bark beetles. Adults fly to mature basidiocarps while larvae live under the bark of trees (Setsuda, 1995). Its ecological counterpart in North America, *Temnochila chlorodia*, is believed to function in spore dispersal of *C. volvatus*, as it lives in but also leaves mature basidiocarps (Borden and McClaren, 1970).

The available information on the ecology and natural history of the three remaining species found in this study (*P. japonicus*, *M. antennatus*, and *G. ipsoides*) is limited. *Pediacus japonicus* has been poorly studied, but the genus *Pediacus* is known to live in association with conifers, usually under the bark of dead trees (Thomas, 2004). If *P. japonicus* frequently moves between *C. volvatus* and under the bark of the tree, the beetle could easily be a vector for spore transport. *Mycetophagus antennatus* is common in Korea and is known to be associated with the fruiting bodies of many Basidiomycota species (Jung and Park, 2013). Lastly, *G. ipsoides* is in the family as *A. suturalis* (Nitidulidae), again known to have a close association with fungi, both feeding on and dispersing the fungi. A related species in Canada, *G. quadrisignatus*, has been shown to be a vector of ear rot of corn, transmitting conidia and ascospores of *Gibberella zeae* (Attwater and Busch, 1983). Since we cannot demonstrate a clear interaction between these coleopteran species and *C. volvatus*, further studies on their ecology is needed.

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