Korean Journal of Microbiology (2018) Vol. 54, No. 4, pp. 362-368
DOI https://doi.org/10.7845/kjm.2018.8079

pISSN 0440-2413
eISSN 2383-9902

Cellulose degrading basidiomycetes yeast isolated from the gut of grasshopper in Korea

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한국의 메뚜기의 장에서 분리된 Cellulose를 분해하는 담자균 효모

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(Received November 5, 2018; Revised November 20, 2018; Accepted November 20, 2018)

Grasshoppers play vital role in the digestion of photosynthetically fixed carbons. With the aid of intestinal microflora, the grasshopper can degrade leaves constituents such as cellulose and hemicellulose. The purpose of this study was to examine cellulolytic yeast isolates from the gut of grasshoppers collected in Gyeonggi Province, South Korea. Among the yeast isolates, ON2, ON17 (two strains), and ON6 (one strain) showed positive cellulolytic activity in the CMC- plate assay. The sequence analyses of D1/D2 domains of the large subunit rDNA gene and the internal transcribed spacer (ITS) regions revealed that the strains ON2 and ON17 were most closely related to *Papiliotrema aspenensis* CBS 13867^T (100%, sequence similarity in D1/D2 domains; 99.4% sequence similarity in ITS) and strain ON6 related to *Saitozyma flava* (100% in D1/D2 domains; 99.0% in ITS). All these three yeast strains are capable of degrading cellulose; therefore, the members of endosymbiotic yeasts may produce their own enzymes for carbohydrate degradation and convert mobilized sugar monomers to volatile fatty acids. Thus, the endosymbiotic yeast strains ON2, ON17 (represents the genus *Papilioterma*) and ON6 (*Saitozyma*) belonging to the family *Tremellomycetes*, are unreported strains in Korea.

Keywords: *Papiliotrema* sp., *Seitozyma* sp., rRNA, grasshopper, yeast

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Pectin and cellulose are the main constituents of plant cell walls, the insects like grasshopper secrete cell wall degrading enzymes to digest the food materials. Some species of grasshoppers feed exclusively on forbs, some eat only grasses, and some eat both forbs and grasses (Joern, 1985). Grasshopper gut consists of foregut, caeca (attached at the beginning of the midgut), midgut and ileum (hindgut), the gut lumen of all grasshoppers was oxidizing and ranged from slightly acid to neutral. Although gut physicochemical conditions may differ among species, they were independent of diet breadth and the percentage of forbs in the diet (Joern and Appel, 1998). Thus, the gut pH of grasshoppers is more favorable for the yeast growth.

Yeasts are collectively capable of utilizing many different substrates, including mono-, di-, and trisaccharides, polyols, ethanol, and organic acids as defined by Lodder (1970). Some yeasts can utilize polysaccharides, but the attack is usually limited to soluble starch, inulin, and more rarely pectin (Ingram, 1958) and reports of yeasts attacking cellulose was less. The first reports on cellulolytic activity was showed by *Trichosporon* species. Cellulase production gaining importance due to several potential applications, such as the production of bioenergy, biofuels and bioethanol as well as application in the textile and paper industries (Zhou *et al*., 2008). A wide variety of microorganisms in nature capable of producing cellulases; some are known as truly cellulolytic, which are capable of degrading natural cellulose. Many bacteria and fungi can degrade cellulose, the final products are generally carbon dioxide and methane when grown in carbohydrate containing media. Only few fungi can convert natural in soluble cellulose to soluble sugar (Goldbeck *et al*., 2012). Currently, there is great interest in the discovery of microbial species that are not known till now as an interesting producer of inputs to industry. By using microbes in biotechnological processes can replace or skip many chemical processes.

During our investigation of cellulolytic microflora in the earthworm gut, yeast strains were isolated and screened for cellulase activity. Among them, ON2 and ON17 belongs to the genus *Papilioterma* and ON6 belongs to the genus *Saitozyma* showed positive to cellulose degradation. Polyphasic characteristics of these strains were also examined. This is the first report on these yeast strains in Korea.

Materials and Methods

Yeast isolation and phenotypic characterization

The grasshoppers are collected, and their bodies were frozen separately at -20°C. On the day of dissection, frozen grasshoppers taken out from the freezer and immediately rinsed with 70% ethanol. The exoskeleton of each grasshopper was then cut along the side and the digestive system was extracted. Whole guts were then stored in 1.5 ml microcentrifuge tubes, the tissue was dissected, and the samples were cut into segments of 0.2–0.4 cm without surface-sterilization. Between 10 and 30 tissue segments were then evenly placed in 9 cm diameter petri dishes containing yeast extract-malt extract (YM) agar plates (pH 3.7–5.0) (Difco) supplemented with 0.01% (w/v) chloramphenicol and then incubated at 25ºC in the dark. The colonies appeared on the plates around a period of 3–5 days were transferred to YM broth for growth. The strains were then purified by repeated streaking of an isolated colony onto YM agar followed by incubation at 25ºC. The plates were incubated at 4ºC, and all colonies were streaked for purification. Purified yeast strains were suspended in YM broth supplemented with 10% glycerol (v/v) and maintained at -80ºC. Morphology, physiology, and biochemical studies were performed following the methods described by Kurtzman *et al*. (2011). For morphology the cells are taken from 3-day-old colonies grown in YM agar (Difco) at 25ºC, the cells were observed using Nikon light microscope (× 1,000 magnification). The size of the cell is measured by a scale bar. Formation of pseudohyphae and true hyphae was investigated by cultivation on potato dextrose agar (PDA, Difco) in the slide culture at 25ºC for 2 weeks, the results observed using Nikon light microscope (× 1,000 magnification). Growth at various temperatures and NaCl concentrations was determined by cultivation in YM agar.

Solidified CMC plate–based clearing assays

Screening of cellulolytic degrading yeast strains using CMC (Carboxymethyl cellulose) plate-based clearing assay was used as described by Johnsen and Krause (2014) with some modified steps. Czapek-Dox-CMC medium containing KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_20$ (0.5 g/L), KCl (0.5 g/L), $FeSO_4 \cdot 7H_2O$

(0.01 g/L), CMC (30 g/L), $NaNO_3$ (2 g/L), noble agar (20 g/L), and antibiotics (0.02%) were poured into petri dishes and was allowed to polymerize at room temperature overnight. The yeast strains were streaked onto the plates. All plates were incubated at 27°C for 12−16 h after which hydrolysis zones were visualized by flooding of the plates/wells with Gram's iodine (2 g potassium iodide and 1 g iodine in 300 ml water) for 5 min followed by a rinse with deionized water. Plates where CMC was omitted (non-substrate) were used as controls in all experiments. All the chemicals were purchased from Difco.

DNA sequencing and phylogenetic analysis

Yeast DNA was extracted and purified using the CTAB method (Cubero *et al*., 1998). The ITS (ITS1−5.8 S−ITS2) region of the rRNA gene was amplified with the primers ITS1F and ITS4 as described by White *et al*. (1990). Amplification of the ITS region was performed using the following conditions: 95ºC for 3 min, followed by 37 cycles of 94ºC for 30 sec, 52ºC for 30 sec, and 72ºC for 30 sec; and a final extension at 72ºC for 10 min. The D1/D2 domain was amplified with the primers NL1 and NL4 as described by Kurtzman and Robnett (1998). Amplification of the D1/D2 domain was performed as follows: 94ºC for 6 min, followed by 40 cycles of 94ºC for 60 sec, 50ºC for 60 sec, and 72ºC for 60 sec; and a final extension at 72ºC for 5 min.

The phylogenetic analysis was aligned with the program CLUSTAL X 1.83 (Thompson *et al*., 1997). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 7 (Kumar *et al*., 2016) and evolutionary distances were calculated using the neighbour-joining method with Kimura's two-parameter distance measure. The confidence levels of the clades were estimated through bootstrap analysis (1,000 replicates) (Felsenstein, 1985).

Results and Discussion

Screening of cellulolytic activities in yeast strains

During the screening of cellulolytic yeast, the strains ON2, ON17, and ON6 showed positive for cellulose degradation by showing clearance area on the plate (Fig. 3). Therefore, the yeast strains utilized cellulose from the medium. These plates provided advantage of using smaller sample volumes for getting a measurable clearance. Cellulolytic activity can be quantified by a variety of methods that have been summarized in papers (Percival Zhang *et al*., 2009). Previously, crystalline cellulose was used but the degradation rates were very slow, most assays were adapted to use more easily degradable soluble cellulose derivatives like carboxymethylcellulose (CMC) (Yeoh *et al*., 1985). Screening for extracellular cellulase production by bacteria and fungi is often done on agar plates containing CMC as substrate (Dashtban *et al*., 2010). This method is popular because the results can be monitored and compared directly and quickly. Over the decades, a variety of dyes have been introduced for this differential staining, the most common of which is Gram's iodine (Kasana *et al*., 2008). Considering the screening plate assay the strains ON2, ON17, and ON6 are selected as a potential producer of cellulase, which showed hydrolytic activity on Carboxymethyl cellulose (CMC).

Morphological characteristics of ON2, ON17, and ON6

In YM broth after 2 days at 25°C, cells of ON2 and ON17 are ovoid to elongate (5.7−6.0 × 3.5−5.0 µm and 5.5−6.3 × 3.7−5.5 µm) and they occur in single or pairs (Fig. 1A and B). The colonies of ON2 and ON7 showed smooth, cream in colour with an entire margin. In YM broth after 2 days at 25ºC, the cells of ON6 are ovoid (5.5−6.0 × 2.0−3.5 µm), they occur in single or pairs (Fig. 1C). The colonies of ON6 are smooth, merged, cream in color with entire margin. Pseudohyphae were absent in all the three strains. Growth of ON2, ON7, and ON6 observed at 15°C, 25°C, and 37°C but not at 40°C. These three strains can reproduce by polar budding.

Species identification and phylogenetic tree analysis

Based on the construction of phylogenetic tree using neighbor joining method, showed the strains ON2 and ON17 clustered with the type strains of *Papiliotrema aspenensis* CBS 13867^T and ON6 clustered with the type strain *Saitozyma flava* CBS 331^T (Fig. 2). Further, the BLASTN (Altschul *et al*., 1997) sequence analysis of the nucleotide D1/D2 domain of the LSU rDNA gene and ITS region of strains ON2 and ON17 with the closest phylogenetic relatives retrieved from the GenBank

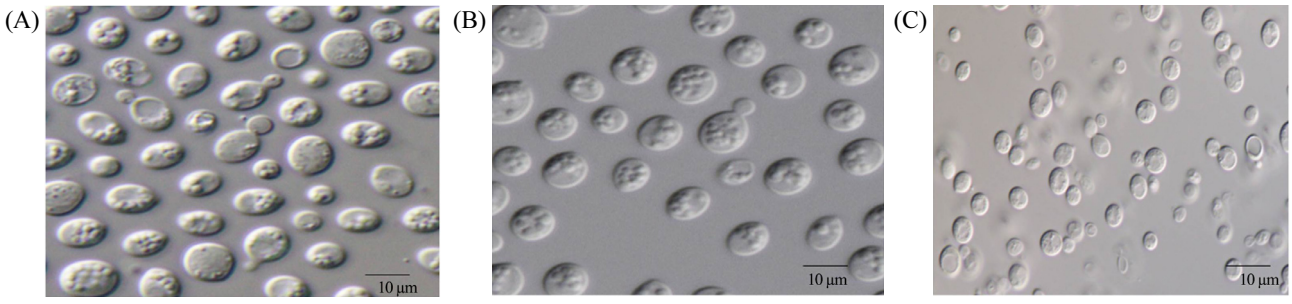

Fig. 1. Cell morphology on YM agar after 3 days at 25°C. (A) ON2, (B) ON17, and (C) ON6. Scale bar, 10 μm.

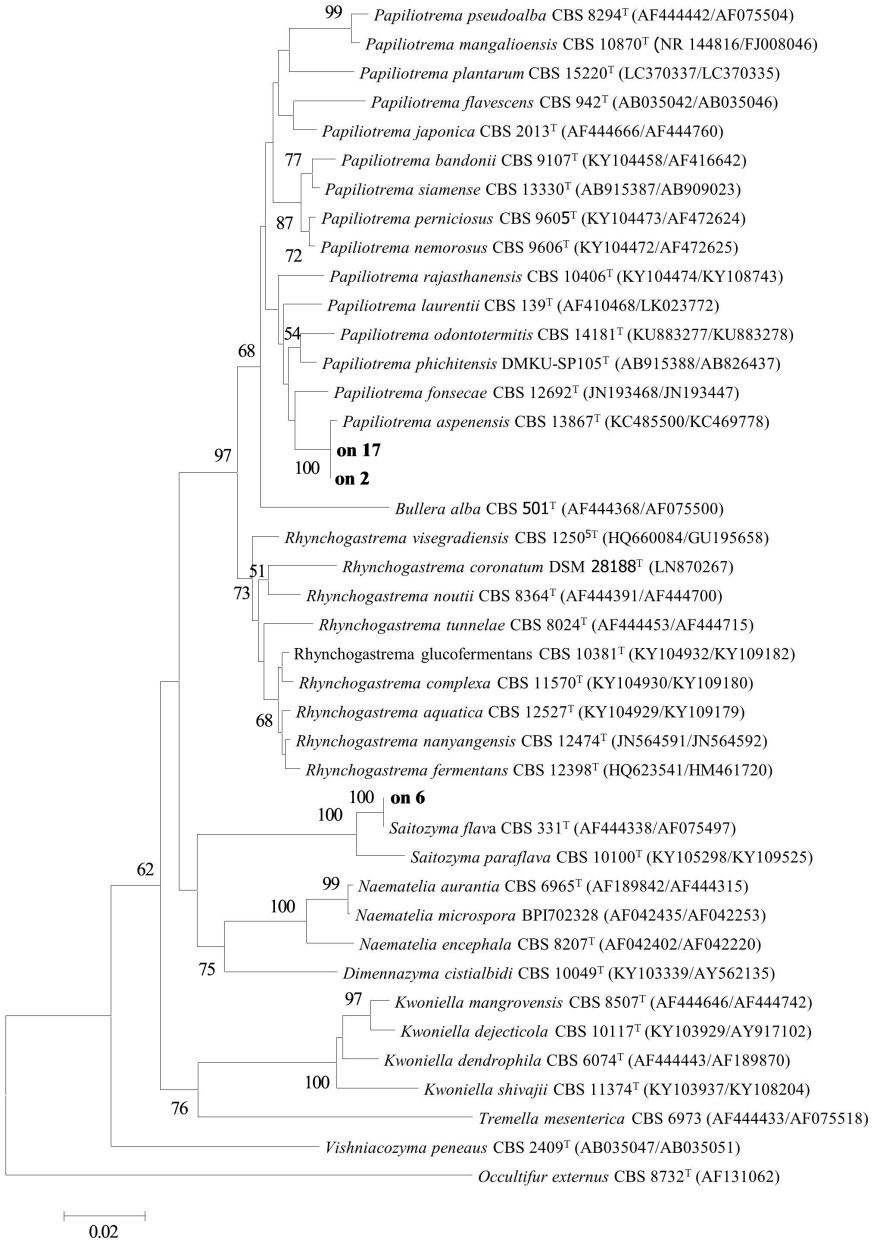

Fig. 2. Phylogenetic tree based on the concatenated sequences of the ITS regions and LSU D1/D2 domain of strain ON2, ON17, and ON6 with the members of closely related taxa. Bootstrap values (> 70%) based on 1,000 replications are shown at the branch nodes. Bar, 0.02 substitutions per nucleotide position is used as an outgroup.

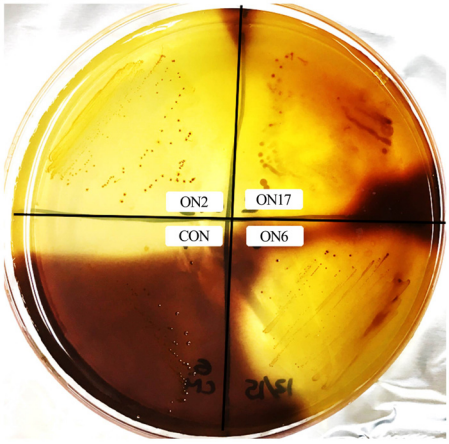

Fig. 3. CMC-plate assay showing cellulolytic activities of strains ON2, ON17, and ON6 with control. Grams Iodine stain was used to visualize the clearance (cellulose degradation) in the plate.

database, showed 100% (D1/D2 domain) and 99.4% (ITS with 1 nucleotide substitution) sequence similarity with *Papiliotrema aspenensis* CBS 13867^{T}, whereas, strain ON6 showed 100% (D1/D2 domain) and 99% (ITS with 5 nucleotide substitution) with the *Saitozyma flava* CBS 331^{T}. Using ITS and LSU barcodes, we were also able to show that strains ON2 and ON17 belongs to the species *Papiliotrema aspenensis* and the strain ON6 belong to the species *Saitozyma flava.* These strains are the unrecorded yeast isolates in Korea.

Description of strains ON2 and ON17 (*Papiliotrema aspenensis*)

In YM broth after 2 days at 25°C, cells of ON2 and ON17 are ovoid to elongate (5.7–6.0 × 3.5–5.0 µm and 5.5–6.3 × 3.7–5.5 µm) and they occur in single or pairs. In YM agar at 25°C the colonies of ON2 and ON7 are smooth, cream in colour with an entire margin (after grown for 3 days). Absence of pseudohyphae formation. Fermentation is negative. L-arabinose, D-arabinose, galactitol, galactose, D-glucitol, D-gluconate, gluconolactone, glucose, D-glucosamine, lactose, maltose, melezitose, melibiose, D-mannitol, raffinose, L-rhamnose, ribitol, D-ribose, salicin, sucrose, trehalose, xylitol, and D-xylose are assimilated as a carbon source. *N*-Acetyl-D-glucosamine, cellobiose, ethanol, erythritol, glycerol, methyl-α-D-glucoside, and myo-inositol are weakly utilized. However, citrate, DL-lactate, methanol, L-sorbose, and inulin was not utilized. Cadaverine dihydrochloride, potassium nitrate, and lysine are utilized as a nitrogen source and sodium nitrate was not. Growth on YM agar with 10% sodium chloride is negative. Growth in vitamins free medium is positive. Growth in 50% glucose/yeast extract (0.5%) is positive. Starch-like substance is produced in the medium. In 100 µg cycloheximide/ml growth is positive. Reaction with diazonium blue B is negative. The major ubiquinone is Q10. The detailed biochemical result similarities between the strains ON2, ON17 with *Papiliotrema aspenensis* was given in the Table 1.

Table 1. Physiological and biochemical tests capable of showing similarity between strains of ON2, ON17, and *Papilioterma aspenensis*

Characteristic	1	2	3
L-Arabinose	+	+	+
D-Arabinose	+	+	+
Cellobiose	w	w	+
α-Methyl-D-glucoside	w	w	+
Raffinose	+	+	+
D-Ribose	+	+	+
Lactose	+	+	+
Starch	-	-	ND
Sucrose	+	+	ND
Trehalose	+	+	+
Maltose	+	+	+
D-Glucosamine	+	+	ND
Melizitoze	+	+	+
Galactitol	+	+	+
Galactose	+	+	+
L-Rhamnose	+	+	+
DL-Lactate	-	-	+
Erythritol	-	-	+
Glycerol	-	-	ND
D-Mannitol	+	+	+
Sodium nitrate	-	w	ND

Species 1, ON2; 2, ON17; 3, *Papilioterma aspenensis*
Growth reactions: -, no growth; w, weak growth; +, strong growth; ND, not determined
Papilioterma aspenensis - data obtained from Ferreira Paim *et al.* (2014).

Description of strain ON6 (*Saitozyma flava*)

In YM broth after 2 days at 25ºC, the cells of ON6 are ovoid (5.5–6.0 × 2.0–3.5 µm), they occur in single or pairs. The colonies of ON6 are smooth, merged, cream in color with entire margin after grown for 3 days in YM agar at 25ºC. Absence of pseudohyphae formation. Fermentation is negative. Glucose,

Table 2. Physiological and biochemical tests capable of showing similarity between strains of ON6 and *Saitozyma flava*

Characteristic	1	2
Diazonium blue B reaction	-	-
Assimilation		
L-Arabinose	+	+
D-Arabinose	-	w
Cellobiose	w	+
α-Methyl-D-glucoside	w	+
Raffinose	+	+
D-Ribose	+	+
D-Glucitol	+	+
Glycerol	-	-
Ethanol	-	-
Lactose	-	+
L-Sorbose	-	-
Sucrose	+	+
Salicin	+	+
L-Rhamnose	+	+
Trehalose	+	+
D-Xylose	+	+
D-Glucosamine	w	w
Galactitol	+	w
Erythritol	+	+
Glycerol	-	-
D-Mannitol	+	+
Quinone	Q10	Q10

Species 1, ON6; 2, *Saitozyma flava*
Growth reactions: -, no growth; w, weak growth; +, strong growth.
Saitozyma flava data obtained from Fonseca *et al.* (2008).

sucrose, raffinose, trehalose, salicin, L- rhamnose, D-xylose, L-arabinose, D-ribose, erythritol, xylitol, galactitol, D-glucitol, D-gluconate, glucanolactone, and melezitose are assimilated as a carbon source. Melibiose, galactose, maltose, methyl-α-D-glucoside, cellobiose, ethanol, D-glucosamine, and *N*-Acetyl-D-glucosamine are weakly assimilated. However, inulin, lactose, soluble starch, L-sorbose, D-arabinose, methanol, glycerol, ribitol, D-mannitol, myo-inositol, DL-lactate, and citrate are not assimilated. Nitrogen sources such as potassium nitrate and L-lysin are assimilated. Production of starch like substance is positive. Growth with 50% glucose is negative. In 100 μg cycloheximide/ml growth is positive. Reaction with diazonium blue B is negative. The major ubiquinone is Q10. The biochemical data between ON6 and *Saitozyma flava* was given in Table 2.

Consequently, from a total number of yeast strains isolated from the gut of grasshopper, the strain ON2, ON17, and ON6 showed clearance of cellulose substrate in the medium. Considering the screening plate assay the strains ON2, ON17, and ON6 are selected as a potential producer of cellulase, which showed hydrolytic activity on Carboxymethyl cellulose (CMC). The yeast strains were identified as (ON2 and ON17) *Papiliotrema aspenensis* and (ON6) *Saitozyma flava* based on polyphasic approach. This is the first report on these unreported species in Korea showing cellulolytic activity.

Nucleotide sequence accession number and culture deposition

The accession numbers of D1/D2 domain of ON2, ON17, and ON6 were MH88019, MH880192, and MK182934. The accession number of ITS regions of ON2, ON17, and ON6 were MH880194, MH880193, and MK182935. The strains are deposited in Korean Collection for Type Cultures, Korea. The KCTC numbers for the strains were ON2 (= KCTC27805), ON17 (= KCTC27806), and ON6 (= KCTC27799).

적 요

메뚜기는 광합성으로 고정된 탄소의 소화에서 중요한 역할을 한다. 장내 미생물 군의 도움으로, 메뚜기는 셀룰로오스 및 헤미셀룰로오스와 같은 잎의 성분을 분해할 수 있다. 본 연구는 한국의 기도에서 수집한 메뚜기 껍질에서 추출한 셀룰로오스 분해 효모 균주를 조사하기 위해 이루어졌다. 효모 균주 중 ON2와 ON17 (두 균주)과 ON6 (한 균주)는 CMC-플레이트 분석에서 셀룰로오스 활성을 보였다. Large subunit rDNA 의 D1/D2 영역의 서열과 internal transcribed spacer (ITS) 영역의 분석 결과, ON2와 ON17 균주가 *Papiliotrema aspenensis* CBS 13867^{T}와 가장 밀접하게 관련되어 있었고(D1/D2 영역의 서열 유사성은 100% ITS에서 99.4%의 서열 유사성) ON6 균주는 *Saitozyma flava*와 관련된(D1/D2 영역에서 100%, ITS에서 99.0%) 밀접하게 관련이 있었다. 이 세 가지 효모 균주는 모두 셀룰로오스를 분해할 수 있으므로 공생하는 효모들은 탄수화물 분해를 위한 효소를 자체적으로 생산하고 당 단당체를 휘발성 지방산으로 전환시킬 수 있다. *Tremellomycetes*에 속하는 공생 효모 균주인 ON2, ON17 (*Papilioterma* 속)과 ON6

(*Saitozyma*속)은 한국에는 보고되지 않은 균주이다.

Conflict of Interest

All authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Acknowledgements

This work was supported by a research grant from Seoul Women's University (2018) and a grant from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea (NIBR2018 39201, 2018 Graduate Program of Undiscovered Taxa).

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