Gibbsiella papilionis sp. nov., isolated from the intestinal tract of the butterfly *Mycalesis gotama*, and emended description of the genus *Gibbsiella*

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A novel Gram-negative, non-motile, facultative anaerobic and rod-shaped bacterium, designated strain LEN33^T, was isolated from the intestinal tract of a butterfly (*Mycalesis gotama*). Strain LEN33^T grew optimally at 37 °C in the presence of 1 % (w/v) NaCl and at pH 9. The novel strain was oxidase-negative and catalase-positive. The major cellular fatty acids were $C_{14:0}$, $C_{16:0}$ and cyclo-$C_{17:0}$. Strain LEN33^T contained two unidentified lipids, three unidentified amino-phospholipids, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). The major isoprenoid quinone was ubiquinone-8 (Q-8). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain LEN33^T was most closely related to *Gibbsiella quercinecans* FRB 97^T and *Gibbsiella dentisursi* NUM 1720^T, with 98.7 % similarities. DNA–DNA hybridization experiments indicated less than 40.7 ± 2 % relatedness to the closest phylogenetic species, *G. quercinecans* FRB 97^T. The G+C content of genomic DNA was 58.7 mol%. Phenotypic, phylogenetic and genotypic analysis indicated that strain LEN33^T represents a novel species within the genus *Gibbsiella*, for which the name *Gibbsiella papilionis* is proposed. The type strain is referred to as LEN33^T (=KACC 16707^T=JCM 18389^T). An emended description of the genus *Gibbsiella* is also proposed.

Insects are the most abundant group of metazoans (May & Beverton, 1990). Researchers estimate that 1.25 million to 10 million species of insects exist, potentially representing over 90 % of all animals (Erwin, 1982, Vilmos & Kurucz, 1998). Due to the size of the insect population, most of the micro-organisms in their gastrointestinal tracts (GIT) are unidentified. These underexplored microbiota could be used in the development of antibiotics or other medications (Fischbach & Walsh, 2009). Identifying the bacteria in the GIT of insects might help prevent disease in the human population (Scully & Bidochka, 2006). The bacterium described below was isolated from the intestinal tracts of a butterfly during an investigation of the microbial ecology of insects in Korea.

The genus *Gibbsiella*, which belongs to the family *Enterobacteriaceae*, was first introduced by Brady *et al.* (2010). At present, the genus contains two species: *Gibbsiella quercinecans* (Brady *et al.*, 2010) and *Gibbsiella dentisursi* (Saito *et al.*, 2012). *G. quercinecans* and *G. dentisursi* are Gram-negative facultative anaerobic and immotile bacteria. *G. quercinecans* was isolated from diseased oak trees and *G. dentisursi* was isolated from the oral cavity of a bear. In this paper, we described a novel strain of a species of the genus *Gibbsiella*, designated LEN33^T, based on the results of physiological, biochemical and genotypic investigations.

Strain LEN33^T was isolated from the intestinal tract of a Chinese bushbrown butterfly (*Mycalesis gotama* Moore) at Seoul, Korea. Homogenized intestine tract tissue was serially diluted with filtered PBS buffer (Bioneer, Korea) and spread on trypticase soy agar (TSA; Bacto) plates. This plate was incubated at 25 °C for 1 week, and a pure culture was obtained by repeated cultivation of single colonies. All tests were conducted in triplicate. For the phylogenetic analysis, the 16S rRNA gene sequence of strain LEN33^T were amplified by colony PCR using PCR pre-mix (iNtRon Biotechnology) with two universal bacterial primers: forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGYTACCTTG-TTACGACTT-3′) (Lane, 1991). Conditions for colony PCR were as follows: Initial denaturation at 94 °C for 30

Abbreviations: DDH, DNA–DNA hybridization; GIT, gastrointestinal tract.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Gibbsiella papilionis* LEN33^T is JQ650257. The GenBank/EMBL/DDBJ accession numbers for the *rpoB* and *gyrB* gene sequences reported in this paper are JX863072 and JX863073, respectively.

Two supplementary figures and a supplementary table are available with the online version of this paper.

min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 1 min 30 s, with a final extension step at 72 °C for 10 min. The 16S rRNA gene amplicon was sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's instructions. The analysis was conducted using an automated system (PRISM 3730XL DNA analyser; Applied Biosystems). The almost complete 16S rRNA gene sequences of the isolate were assembled using SeqMan (DNASTAR). The assembled sequence was compared with other sequences of type strains in the EzTaxon-e server (Kim *et al.*, 2012). The results of the 16S rRNA gene sequence similarity analysis showed that strain LEN33^T was related to *G. quercinecans* FRB 97^T (98.7 % similarity) and *G. dentisursi* NUM 1720^T (98.7 % similarity). For detailed phylogenetic description, multilocus sequence analysis was performed. The protein-encoding genes *gyrB* (DNA gyrase subunit *β*) and *rpoB* (RNA polymerase subunit *β*) were amplified and sequenced, as described by Brady *et al.* (2008). The 16S rRNA gene and protein-encoding genes sequences of LEN33^T and those of closely related species were aligned using the multiple alignment programme CLUSTAL W (Thompson *et al.*, 1994). The aligned sequences were checked manually using BioEdit software (Hall, 1999). The phylogenetic trees based on the 16S rRNA, *gyrB* and *rpoB* gene sequences of strain LEN33^T and its relatives were reconstructed using the MEGA5 software program (Tamura *et al.*, 2011). Phylogenetic correlations were ascertained using neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) methods with 1000 bootstrap replicates. The phylogenetic trees based on the 16S rRNA gene and protein-encoding genes sequences showed that the isolate formed a cluster with other members of the genus *Gibbsiella* (Fig. 1 and Fig. S1 available in IJSEM Online). In order to perform a more comprehensive characterization of strain

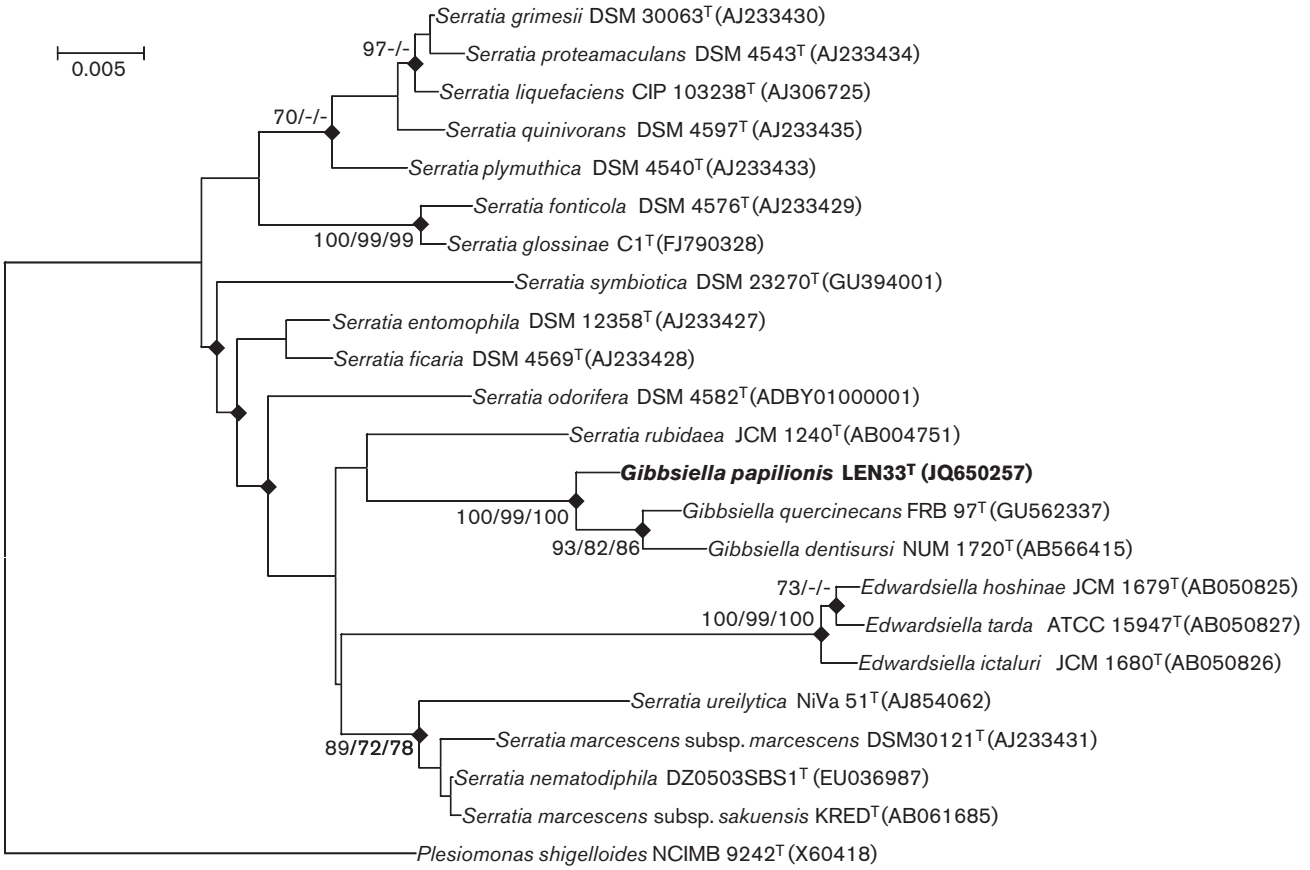

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparisons, reconstructed using the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms. Filled diamonds represent identical branches that are present in phylogenetic consensus trees reconstructed using the three different methods. Numbers at nodes indicate bootstrap values as percentages of 1000 replicates. Values lower than 70 % are not shown at the branch points. Bar, 0.005 accumulated changes per nucleotide.

LEN33^T, *G. quercinecans* FRB 97^T (=LMG 25500^T) was obtained from the Belgian Co-ordinated Collections of Micro-organisms/Laboratorium voor Microbiologie, Universiteit Gent (BCCM/LMG).

Growth under anaerobic conditions was determined after 7 days of cultivation at 37 °C in an anaerobic chamber filled with an atmosphere composed of N_2:CO_2:H_2 at a ratio of 90 : 5 : 5. The growth test was performed at different temperatures (4, 15, 20, 25, 30, 37, 45, 55 and 65 °C) on trypticase soy broth (TSB; Bacto) in triplicate. Salt requirement and tolerance were tested under different concentration of NaCl (0, 1, 2, 3, 4, 5, 8, 10, 12 and 15 %, w/v) in a medium that comprised all of the ingredients of TSB except NaCl. Growth at various pH (4.0–10.0 at intervals of 1.0 pH unit) was tested on TSB at 37 °C. The pH of each medium was adjusted with 50 mM MES for pH 4–6, 50 mM TAPS for pH 7 and 8 and 50 mM Na_2HPO_4 for pH 9 and 10. The OD_{600} of each culture was measured using a spectrophotometer (SYNERGY MX; BioTek) after 24 h, 48 h and 7 days of incubation. These tests demonstrated that LEN33^T grew at 4–37 °C in 0–5 % (w/v) NaCl at a pH 5–9. Optimal growth conditions for the isolate were 37 °C, a salinity of 1 % (w/v) NaCl and pH 8–9. Unless stated otherwise, all experiments were performed under optimal growth conditions for 48 h incubation time. Light microscopy was used to observe Gram-staining and cell morphology (ECLIPSE 50*i*; Nikon). Gram-staining was carried out with a Gram-staining kit (bioMérieux) according to the manufacturer's instructions. Motility tests were conducted in semi-solid TSA (containing 0.4 % agar) (Tittsler & Sandholzer, 1936). The isolate was Gram-negative, rod-shaped and non-motile. Strain LEN33^T formed white–cream-coloured, circular, smooth and convex colonies after 24 h on TSA medium at 37 °C.

Catalase activity was identified by bubble production in 3 % (v/v) hydrogen peroxide. Oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux). Enzyme activities of the isolate were ascertained using API ZYM test strips (bioMérieux) and API 20NE test strips (bioMérieux), according to the manufacturer's instructions. Sole carbon source assimilation and acid production from carbohydrates were tested using GN2 MicroPlates (Biolog) with GN/GP inoculating fluid (Biolog) and API 50 CH test strips (bioMérieux) with 50 CHB/E medium (bioMérieux), respectively, according to the manufacturer's instructions. The isolate differed from *G. quercinecans* FRB 97^T by the ability to assimilatie α-cyclodextrin, glycogen, *N*-acetyl-D-galactosamine, adonitol, i-erythritol, xylitol, acetic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, α-ketobutyric acid, glucuronamide, L-alanine, L-alanylglycine, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, D-serine, urocanic acid, thymidine and DL-α-glycerol phosphate (determined using GN2 MicroPlate); and enzyme activity level for cystine arylamidase (API ZYM). The complete results of biochemical tests and the

Table 1. Differential characteristics of strain LEN33^T and the closest related species

Strains: 1, LEN33^T; 2, *G. quercinecans* LMG 25500^T. All data were from the current study except where indicated. Data for carbon source assimilation, acid production from carbohydrate and enzyme activity were obtained with a GN2 MicroPlate (Biolog), API 50CH and API 20NE/API ZYM, respectively. All strains were positive for catalase and negative for oxidase. All strains assimilated dextrin, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, L-arabinose, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, *myo*-inositol, α-lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, β-methyl-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, pyruvic acid methyl ester, *cis*-aconitic acid, citric acid, D-gluconic acid, α-ketoglutaric acid, DL-lactic acid, succinic acid, bromosuccinic acid, succinamic acid, L-asparagine, L-aspartic acid, L-serine, inosine, uridine, glycerol, α-D-glucose-1-phosphate and D-glucose-6-phosphate. All strains produced acids from glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, D-sorbitol, methyl-α-D-glucoside, *N*-acetylglucosamine, arbutin, aesculin, salicin, maltose, melibiose, sucrose, trehalose, raffinose, gentiobiose, turanose, gluconate, 2-ketogluconate and 5-ketogluconate. All strains were positive for alkaline phosphatase, esterase (C4), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase (API ZYM), potassium nitrate (reduction of nitrates to nitrites), L-arginine dihydrolase, urease, β-glucosidase (aesculin hydrolysis) and β-galactosidase (ONPG hydrolysis) (API 20NE). +, Positive; −, negative.

Characteristic	1	2
Temperature optimum (°C)	37	30*
Assimilation of:		
α-Cyclodextrin, glycogen, *N*-acetyl-D-galactosamine, adonitol, i-erythritol, xylitol, acetic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, α-ketobutyric acid, glucuronamide, L-alanine, L-alanylglycine, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, D-serine, urocanic acid, thymidine, DL-α-glycerol phosphate	+	−
Acid production from:		
Lactose, inositol, starch, L-fucose	−	+
Enzyme activity		
Cystine arylamidase	+	−

*Data from Brady *et al.* (2010).

characteristics of strain LEN33^T that distinguish it from *G. quercinecans* FRB 97^T are presented in Table 1 and the species description.

Chemotaxonomic analyses were performed with the cell biomass of the isolate and the reference species cultured on TSA plates at 37 °C for 48 h, which were standardized as exponential phase of physiological ages in both species. The protocol of the Sherlock Microbial Identification Systems (MIDI, 1999) was followed to extract fatty acids. Fatty acid composition was analysed by gas chromatography (Agilent 7890 gas chromatograph, Agilent Technologies) and individual fatty acids were identified using the Microbial Identification software package (Sherlock version 4.0) (Sasser, 1990) in combination with the TSBA6 database. The most dominant fatty acids (>10 %) were $C_{16:0}$ (39.11 %), cyclo-$C_{17:0}$ (19.91 %) and $C_{14:0}$ (10.10 %). The complete fatty acid compositions of the isolate and the type strain of *G. quercinecans* are presented in Table S1. The polar lipid composition was determined by extracting polar lipids from the isolate according to the method described by Xin *et al.* (2000) and separating them by two-dimensional TLC on a silica gel 60 F_{254} plate (Merck). The reagents used for detecting and identifying polar lipids were as follows: 10 % ethanolic molybdatophosphoric acid reagent for total lipids, ninhydrin reagent for amino-containing lipids and zinzadze reagent for phospholipids (Tindall, 1990). The phospholipids were identified using one-dimensional TLC with standard compounds (Sigma). The polar lipids of strain LEN33^T included two unidentified lipids (L1–2), three unidentified amino-phospholipids (APL1–3), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) (Fig. S2). Chloroform : methanol (2 : 1, v/v) was used to extract isoprenoid quinone from strain LEN33^T and *G. quercinecans*. Isoprenoid quinone, which was extracted as described by Collins and Jones (1981a), was purified by one-dimensional TLC on a silica gel 60 F_{254} plate (Merck) and analysed by reverse–phase HPLC (Collins & Jones, 1981b) using a Thermo ODS HYPERSIL (250 × 4.6 mm) column. The most dominant isoprenoid quinone of strain LEN33^T was ubiquinone Q-8, which is the dominant quinone in *G. quercinecans, G. dentisursi* (Saito *et al.*, 2012) and related *Enterobacteriaceae* species such as *Serratia marcescens* and *Edwardsiella tarda* (Collins & Jones, 1981a). The isolate also possessed menaquinone MK-8(H_4) as a minor quinone.

The genomic DNAs of strain LEN33^T and *G. quercinecans* were extracted for genotype analyses, as described by Rochelle *et al.* (1992). The G+C content of the genomic DNA was estimated by a fluorimetric method with SYBR Gold I and a real-time PCR thermocycler (BIORAD) (Gonzalez & Saiz-Jimenez, 2002). For calibration, the genomic DNAs of *Escherichia coli* K-12, *Ruegeria pomeroyi* DSS-3^T and *Ruminococcus obeum* ATCC 29174^T were used as a reference for the analysis. The genomic DNA G+C content of the isolate was 58.7 mol%. This value had similarity within the G+C content range of the genus *Gibbsiella* (56.0–58.7 mol%) (Brady *et al.*, 2010). To clarify the genetic relativeness between strain LEN33^T and the type species, DNA–DNA hybridization (DDH) was performed using a genome-probing microarray (Bae *et al.*, 2005, Chang *et al.*, 2008). The DDH values were calculated from the signal-to-noise ratio of the genomic probes (Loy *et al.*, 2005). The value of DDH between the isolate and the type species, which had more than 97 % 16S rRNA sequence similarity, was 41 ± 2 % (30 ± 3 % in reciprocal). The DDH value was below the novel genotypic species threshold of 70 % (Wayne *et al.*, 1987).

The phenotypic, genotypic and phylogenetic analyses suggest that strain LEN33^T represents a novel species of the genus *Gibbsiella*, for which the name *Gibbsiella papilionis* sp. nov. is proposed.

Emended description of the genus *Gibbsiella*

The description of the genus *Gibbsiella* is based on that given previously by Brady *et al.* (2010), but with the following amendment. The DNA G+C content ranges from 56.0 to 58.7 mol%.

Description of *Gibbsiella papilionis* sp. nov.

Gibbseilla papilionis (pa.pi.li.o′nis. L. gen. n. *papilionis* of a butterfly, isolated from the intestinal tracts of a butterfly, *Mycalesis gotama*).

Facultatively anaerobic, Gram-negative, rod-shaped (1.5 μm long and 0.5 μm wide), non-motile, oxidase-negative and catalase-positive. Colonies on TSA medium are circular, opaque with a cream colour, smooth and convex. Growth occurs at temperatures between 4 and 37 °C, 0–5 % (w/v) NaCl and pH 5–9. Optimal growth conditions are pH 8–9, in the presence of 1 % (w/v) NaCl and at 37 °C. Acid is produced from glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, D-sorbitol, methyl-α-D-glucoside, *N*-acetylglucosamine, arbutin, aesculin, salicin, maltose, melibiose, sucrose, trehalose, raffinose, gentiobiose, turanose, gluconate, 2-ketogluconate, 5-ketogluconate. The isolate assimilates α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, L-arabinose, D-arabitol, cellobiose, adonitol, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, *myo*-inositol, α-lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, β-methyl-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, pyruvic acid methyl ester, succinic acid mono-methyl-ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, D-serine, L-serine, γ-amino butyric acid, urocanic acid, inosine, uridine, thymidine, glycerol, DL-α-glycerol phosphate, α-D-glucose-1-phosphate and D-glucose-6-phosphate. Positive for the following enzymes'

activities: alkaline phosphatase, esterase (C4), leucine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase (API ZYM), potassium nitrate (reduction to nitrates to nitrites), L-arginine dihydrolase, urease, β-glucosidase (aesculin hydrolysis) and β-galactosidase (ONPG hydrolysis) (API 20NE). The predominant quinone is Q-8 and minor quinone is MK-8(H_4). The major cellular fatty acids are $C_{14:0}$, $C_{16:0}$ and cyclo-$C_{17:0}$. The polar lipids comprise two unidentified lipids, three unidentified aminophospholipids, phosphatidylethanolamine and phosphatidylglycerol.

The type strain is LEN33^T (=KACC 16707^T=JCM 18389^T), isolated from the intestinal tract of Chinese bushbrown (*Mycalesis gotama* Moore) at Seoul, Korea. The DNA G+C content of the type strain is 58.7 mol%.

Acknowledgements

We thank Dr J. P. Euzéby (École Nationale Vétérinaire, France) for etymological advice, Jaejoon Jung and Professor Woojun Park (Korea University, Korea) for MIDI analysis and Hae-Won Lee and Dr Seong Woon Roh (Jeju Center, Korea Basic Science Institute, Korea) for isoprenoid quinone analysis. This work was supported by grants from the National Research Foundation of Korea and the National Institute of Biological Resources (NIBR) Mid-Career Researcher Program (2011-0028854).

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