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Authors: Zaragoza-Ortega, Monica, Segura-León, Obdulia L., Hernández-Cruz, Julián, Valdez-Carrasco, Jorge, and Sánchez-Soto, Saúl

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The response of *Phyllophaga brevidens* and *Phyllophaga lenis* (Coleoptera: Scarabaeidae) to methyl 2-(methylthio) benzoate and light

Monica Zaragoza-Ortega[1], Obdulia L. Segura-León[1,], Julián Hernández-Cruz[2], Jorge Valdez-Carrasco[1], and Saúl Sánchez-Soto[3]*

Abstract

A sexual attractant of some *Phyllophaga* species (Coleoptera: Scarabaeidae: Melolonthinae), methyl 2-(methylthio)benzoate, was studied alone and in combination with light from a solar-powered light-emitting diode (LED) to determine their comparative attraction potential for *Phyllophaga* species in a sugarcane crop. Four trap treatments were evaluated: sex attractant, sex attractant plus light, light alone, and a control trap without attractant or light. The number and species of *Phyllophaga* captured were determined over two 5 mo adult emergence periods from Mar–Jul in 2014 and 2015. Additionally, the cytochrome oxidase I (*CO1*) gene of the species that responded to the compound was amplified, and a phylogenetic tree was constructed using those sequences and sequences found in GenBank. Two species of *Phyllophaga*, *Phyllophaga brevidens* (Bates) (Coleoptera: Scarabaeidae) and *Phyllophaga lenis* (Horn) (Coleoptera: Scarabaeidae), responded to the compound. The traps with sex attractant plus light treatment captured significantly more beetles than the other treatments. Our results suggest that the combination of methyl 2-(methylthio)benzoate and light can be a relatively selective method for the capture of the males of these species of agricultural importance, and it also attracts some females. Finally, analysis of the sequences of the mitochondrial *CO1* gene showed that the *Phyllophaga* species captured with this compound were phylogenetically related.

Key Words: solar cell; LED; June beetles; May beetles; sugarcane

Resumen

Se evaluó 2-(metiltio)benzoato de metilo, un atrayente sexual,de algunas especies de *Phyllophaga* (Coleoptera: Scarabaeidae: Melolonthinae), solo y en combinación con iluminación de un diodo emisor de luz (LED) impulsado por energía solar, para determinar su capacidad de atracción comparativa para especies de *Phyllophaga* dentro del cultivo de caña de azúcar. Se evaluaron cuatro tratamientos de trampas: un atrayente sexual, un atrayente sexual más luz LED, solo luz y una trampa de control sin atrayente ni luz. Se determinó el numero de especies de *Phyllophaga* capturados por trampa en dos periodos de emergencia de los adultos, de marzo a julio del 2014 y 2015. También, se amplificó el gen *COI* de las especies que respondieron al compuesto y se reconstruyó un árbol filogenético utilizando estas secuencias y secuencias encontradas en el banco de genes. Dos especies del género *Phyllophaga*, *P. brevidens* (Bates) y *P. lenis* (Horn) respondieron al compuesto. Las trampas con atrayente más luz fue el tratamiento que capturo un mayor número de individuos en relación a los otros tratamientos**.** Nuestros resultados sugieren que la combinación de methyl 2-(methylthio)benzoate de metilo y luz, puede ser un método selectivo para el manejo de estas especies de importancia agricola, y la capacidad de captura no solo fue de machos sino también de hembras. Finalmente, el análisis de las secuencias del gen *COI* de la mitocondria, muestra que las especies de *Phyllophaga* que han sido capturadas con este compuesto están filogenéticamente relacionadas.

Palabras Clave: celda solar; LED; escarabajos de junio; escarabajos de mayo; caña de azúcar

The genus *Phyllophaga* (*sensu lato*) (Coleoptera: Scarabaeidae: Melolonthinae), commonly known as May or June beetles as adults and white grubs in the larval stage, are major pests of many crops. Whereas adults attack the foliage, immature stages have a higher economic impact because they feed on the roots and cause wilting and plant death (Riess & Flores 1976; Morón 1986). In Mexico, severe damage caused by white grubs is most frequent in maize and sugarcane crops. In Tamaulipas, Mexico losses in maize production can reach 48% (Villalobos 1998), whereas in Morelos State losses are equivalent to about 32% (Villalobos et al. 2001; Núñez-Valdez et al. 2002).

Use of pheromones for the attraction of Coleoptera is widely known. Numerous compounds are based mainly on isopropenoid derivatives, fatty acids, and amino acids (Tillman et al. 1999). In the case of *Phyllophaga*, Pherobase (2016) (a semiochemical and pheromone database) cites 20 compounds attractive to *Phyllophaga*, including palmitic, linoleic, and stearic acids. Other authors also mention 1-octadecanol, 11-n-decyl tetracosane (Romero-López et al. 2003), methyl ester of L-isoleucine (Zhang et al. 1997; Leal et al. 2003; Nojima et al. 2003), and methyl 2-(methylthio)benzoate (Robbins et al. 2003; Robbins et al. 2006; Robbins et al. 2011; Morales-Rodríguez et al. 2011).

[1]Colegio de Postgraduados, Campus Montecillo, Fitosanidad-Entomología y Acarología, Estado de México 56230, México; E-mail: zaragoza.monica@colpos.mx (M. Z.-O), sleon@colpos.mx (O. L. S.-L.), jvaldez@colpos.mx (J. V.-C.)
[2]Universidad Tecnológica de la Sierra Sur de Oaxaca, Magnolia S/N Villa Sola de Vega Oaxaca 71400, México; E-mail: jhcciidir@yahoo.com.mx (J. H.-C.)
[3]Colegio de Postgraduados, Campus Tabasco, Entomología, Periférico Carlos A. Molina S/N, H. Cárdenas, Tabasco 86500, México; E-mail: ssoto@colpos.mx (S. S.-S.)
*Corresponding author: sleon@colpos.mx (O. L. S.-L.)
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Methyl 2-(methylthio)benzoate was reported by Coca-Abia and Robbins (2006) and Robbins et al. (2011) as a sexual attractant though it attracts more than 1 *Phyllophaga* species. They also suggest that the attraction to this compound could be used to establish phylogenetic relationships among the species that are attracted.

Use of light traps is a common technique for capturing adult Melolonthinae (Alcazar-Ruiz et al. 2003; Aragón-García et al. 2008; Lugo-García et al. 2011; Hernández-Cruz et al. 2014); however, such traps have shortcomings such as their dependence on electricity or batteries. These drawbacks could hinder the use of lights in areas lacking electricity, or when operating costs are affected by daily battery changes. Therefore, use of solar-powered light might be a promising alternative for trapping these pests. Here, we report the effectiveness of the sexual attractant methyl 2-(methylthio)benzoate, alone and in combination with a light source, to capture *Phyllophaga* species in a sugarcane crop. Additionally, the mitochondrial cytochrome oxidase I (*CO1*) gene from the captured insects was used to determine if the species that responded to this compound were closely related.

Materials and Methods

This research was conducted in the town of San Miguel Treinta, Tlaltizapan, Morelos, Mexico, which is located at 18.718500°N and 99.180611°W. The altitude is 990 m asl, and the climate is warm subhumid. Annual average temperature is 23.5 °C; annual rainfall is 840 mm, and the rainy season is from Jun to Oct (INEGI 2010).

The traps were constructed from plastic trays of 5 L capacity and 35 cm diameter. Plastic caps of similar diameter were held with 2.5 cm wide aluminum strips separated by 20 cm. A 12 cm wide hole was made in the center of the plastic caps, and a 1.2 V light-emitting diode (LED) lamp attached to a 5.4 × 4 cm solar cell was inserted. In the traps baited with 1 mg of methyl 2-(methylthio)benzoate (Santa Cruz Biotechnology, Santa Cruz, California) sexual attractant, a rubber septum containing the lure was placed inside at the top of the plastic caps. During the collection period, water with detergent was placed in the trap to retain the collected specimens.

The treatments were methyl 2-(methylthio)benzoate sexual attractant; solar-powered LED light; sexual attractant plus light; and neither sexual attractant or light (control). The response variable was the number of *Phyllophaga* adults collected, by species, for each treatment. The collected specimens were preserved in alcohol for morpho-specific classification, assembly, and identification. The sexual attractant was replenished every 30 d. The experiment had a completely randomized design with 4 treatments and 3 replications. The treatments were evaluated during 2 adult emergence periods from Mar–Jul of 2014 and 2015. Within each emergence period, collections were made on 8 dates. The traps were set in a 1 ha sugarcane field of cultivar MY 55-14. The data were analyzed by analysis of variance (ANOVA) at a 95% confidence level ($\alpha = 0.05$) and Tukey multiple comparison tests ($\alpha = 0.05$) with SAS® software (SAS Institute Inc. 2004).

CO1 GENE AMPLIFICATION

Samples for total DNA extraction was obtained from the thorax and legs of *Phyllophaga lenis* (Horn) (Coleoptera: Scarabaeidae) and *Phyllophaga brevidens* (Bates) (Coleoptera: Scarabaeidae) after morphological identification. The DNA extraction method used was the cetyltrimethylammonium bromide (CTAB 2%) method according to Black & DuTeau (1997). A 658 bp fragment of the *CO1* gene was amplified with LCO1490 forward and HCO2198 reverse primers, designed by Folmer et al. (1994) and proposed as a biological identification system for animals by Hebert et al. (2003). The PCR amplification was performed in a thermocycler (Thermo Scientific®, Waltham, Massachusetts) according to the following program: an initial 95 °C denaturation step for 2 min; 30 cycles of denaturation for 30 s at 95 °C, annealing at 51 °C for 50 s , and extension at 72 °C for 1 min; and a final extension step of 72 °C for 5 min. The PCR amplified products were sent to the company Macrogen (Korea) for sequencing in the forward and reverse direction.

SEQUENCE ANALYSIS

The sequences obtained were assembled with the Sequencher® 5.4.1 software (Gene Codes Corporation, Ann Arbor, Michigan) and aligned with ClustalW in MEGA 6.06 (Tamura et al. 2013). Comparison with other sequences was done with BLASTN (Basic Local Alignment Search Tool) at the nucleotide NCBI database by genus and gene analyzed. Altogether, 29 sequences of the barcode region of the *CO1* gene from the captured *Phyllophaga* species were used for phylogenetic analysis, and *Caryedon gonagra* (F.) KP216604 was used as the outgroup. The amino acid translation was done using the invertebrate's mitochondria genetic code (NCBI-The Genetic Codes); then, before phylogenetic inference from DNA, the probabilities of change between nucleotides was accessed by a nucleotide substitution models test with MEGA 6.06 (Tamura et al. 2013)(free online software) , to reduce biases in the DNA evolutionary changes estimation (Posadas 2009)

The phylogenetic tree was constructed using the maximum likelihood method with the general time reversible (GTR) model, with a gamma distribution, and invariant sites (GTR + G + I) resulted from the nucleotide substitution models test; this means that the data set for the analysis had an unequal base frequency and all pair substitution rates have different frequencies (GTR), with different substitution rates among sites (G) and different proportions of invariables sites (I) (Posadas 2009), and 500 bootstrap replicates (Felsenstein 1985). The sequences generated from this research were deposited at the NCBI Nucleotide GenBank with the accession numbers KX271883 to KX271888.

Results

During the 2 trapping seasons, 349 *Phyllophaga* adults were captured. The sex ratio of the collected insects was 93.4% male and 6.6% female. The species of the females were not determined. Captured males were *P. brevidens* and *P. lenis.* Males of *P. brevidens* were captured more frequently during the trapping, and the treatment with the greatest number of captures was attractant plus light. Males of the both species and a few females were collected in this treatment (Fig. 1, Supplemental Table 1). The numbers of insects collected using the different techniques during the 2 sampling periods were: sex attractant, 53; sex attractant plus light, 280; light, 16; and control, 0. During the 2014 collection period, 255 adult specimens were captured (240 males and 15 females). During the 2015 collection period, only 94 adults were captured.

The ANOVA showed significant differences in beetle captures among treatments. The attractant plus light treatment caught more beetles than the other treatments, and the other treatments were not statistically different (Table 1).

CO1 GENE ANALYSIS

Comparative analysis of the sequences from the collected insects with the BAST search tool at the NCBI database resulted in 27 species and 160 sequences for the mitochondrial *CO1* gene for the genus *Phyllophaga*. Only 2 of the 5 species that have been reported to respond to methyl 2-(methylthio)benzoate were found in the database. The phy-

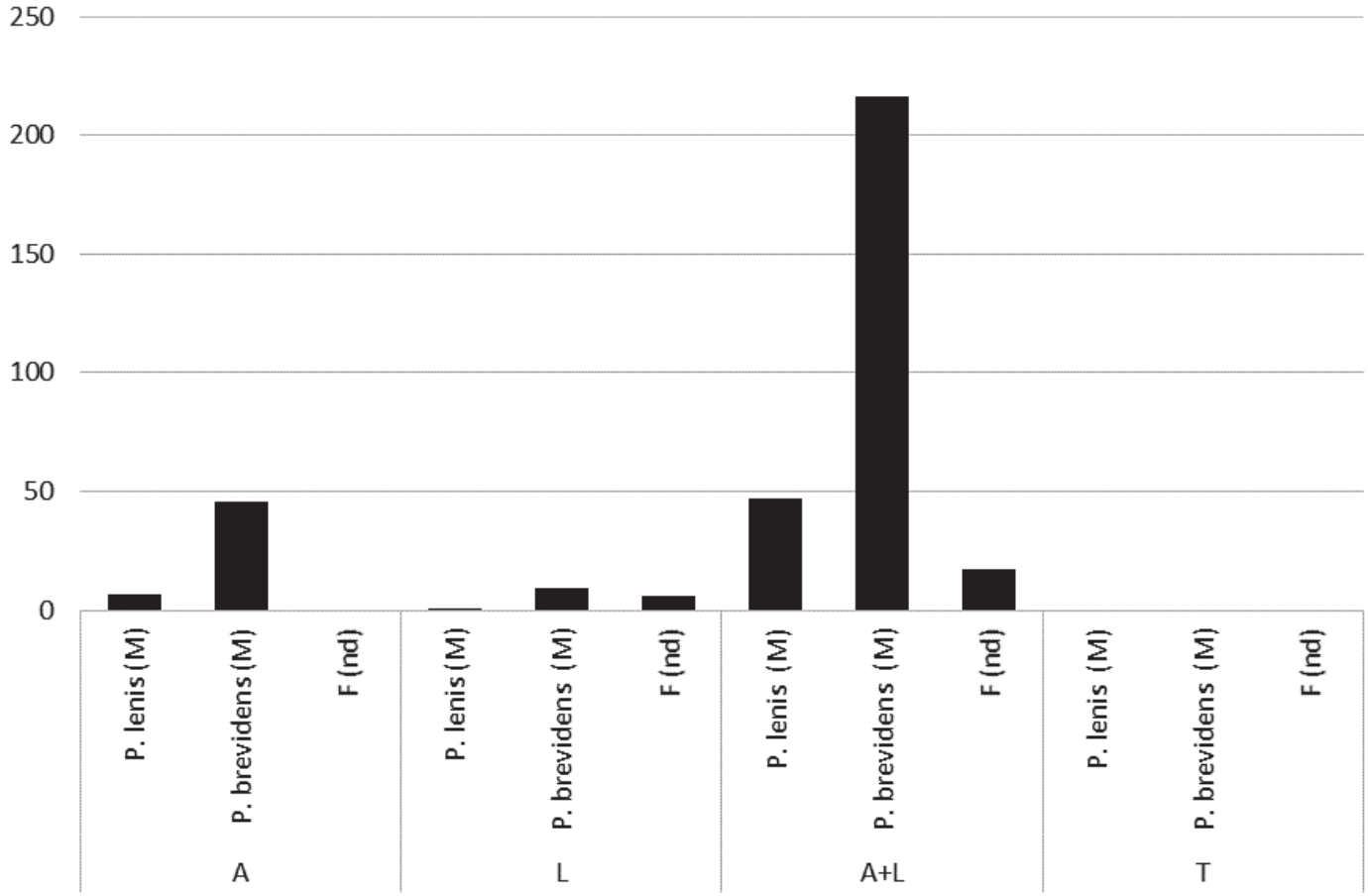

Fig. 1. Numbers of *Phyllophaga brevidens* and *Phyllophaga lenis* adults trapped per species, sex, and treatment during 2 sampling periods in Tlaltizapan, Morelos, Mexico. A = attractant, L= light, C = control, M = males, F = females, nd = not determined.

logenetic tree constructed using the *CO1* gene sequences from the 27 *Phyllophaga* species grouped *P. brevidens* and *P. lenis* with *P. tristis* (F.) (Coleoptera: Scarabaeidae: Melolonthinae) and *P. crinita* (Burmeister) (Coleoptera: Scarabaeidae: Melolonthinae), with a bootstrap value of 92% (Fig. 2). These species belong to the Anodentata group, as described by Horn (1887) and Morón (1986). These results support the hypothesis that the species captured with this compound are phylogenetically related.

Discussion

Methyl 2-(methylthio)benzoate attracted 2 *Phyllophaga* species in Morelos: *P. brevidens* and *P. lenis*. These species can be added to the growing list of *Phyllophaga* species responsive to this sex attractant.

Table 1. Mean numbers of male *Phyllophaga* captured with different traps during 2 sampling periods (Mar–Jul 2014 and 2015) in Tlaltizapan, Morelos.

Trap type	*P. lenis*	*P. brevidens*	Total no. captued
Attractant plus light	0.9792a	4.500a	5.833a
Attractant	0.1458b	0.958b	1.104b
Light	0.0208b	0.188b	0.333b
Control	0.0000b	0.0000b	0.000b

Means within a column followed by the same letter are not significantly different (α = 0.05) by ANOVA and the Tukey multiple comparison test. No: number.

The use of this compound in combination with LED lights increased the number of insects 5 fold as compared to the attractant only. Additionally, a few females were also collected when the lights were incorporated into the traps. The attraction of *Phyllophaga* species females has not been reported by previous researchers that used this attractant. However, similar results have been observed in other species when light and attractants are used together (Monserrat 2008). Hence, the use of solar-powered LED lights might be a useful tool for capturing these species in the crop areas lacking electricity. Therefore, this system can be used as part of integrated pest management.

The response of more than 1 species to this attractant is consistent with results by Robbins et al. (2011) and Morales-Rodriguez et al. (2011). Both studies also reported that this compound attracted more than 1 species. The species caught with this attractant in Morelos, *P. brevidens* and *P. lenis,* are different than reported previously: *Phyllophaga crinita* in Dallas, Texas (Robbins et al. 2003); *P. tristis* in Greensburg, Kansas; *P. apicata* Reinhard (Coleoptera: Scarabaeidae: Melolonthinae) in Gainesville, Texas (Robbins et al. 2011); and *P. menetriesi* (Blanchard) (Coleoptera: Scarabaeidae: Melolonthidae) and *P. lissopyge* (Bates) (Coleoptera: Scarabaeidae: Melolonthinae) in South America (Morales-Rodriguez et al. 2011). All the species captured belong to genus *Phyllophaga*, and most of these species are included in the *anodentata* group (Morón 1986).

The results of this research show that methyl 2-(methylthio)benzoate can be used in combination with solar-powered LED lights to increase the capture of certain *Phyllophaga* spp. Zaragoza-Ortega et al. (2016) reported additional *Phyllophaga* spp. present in the same area

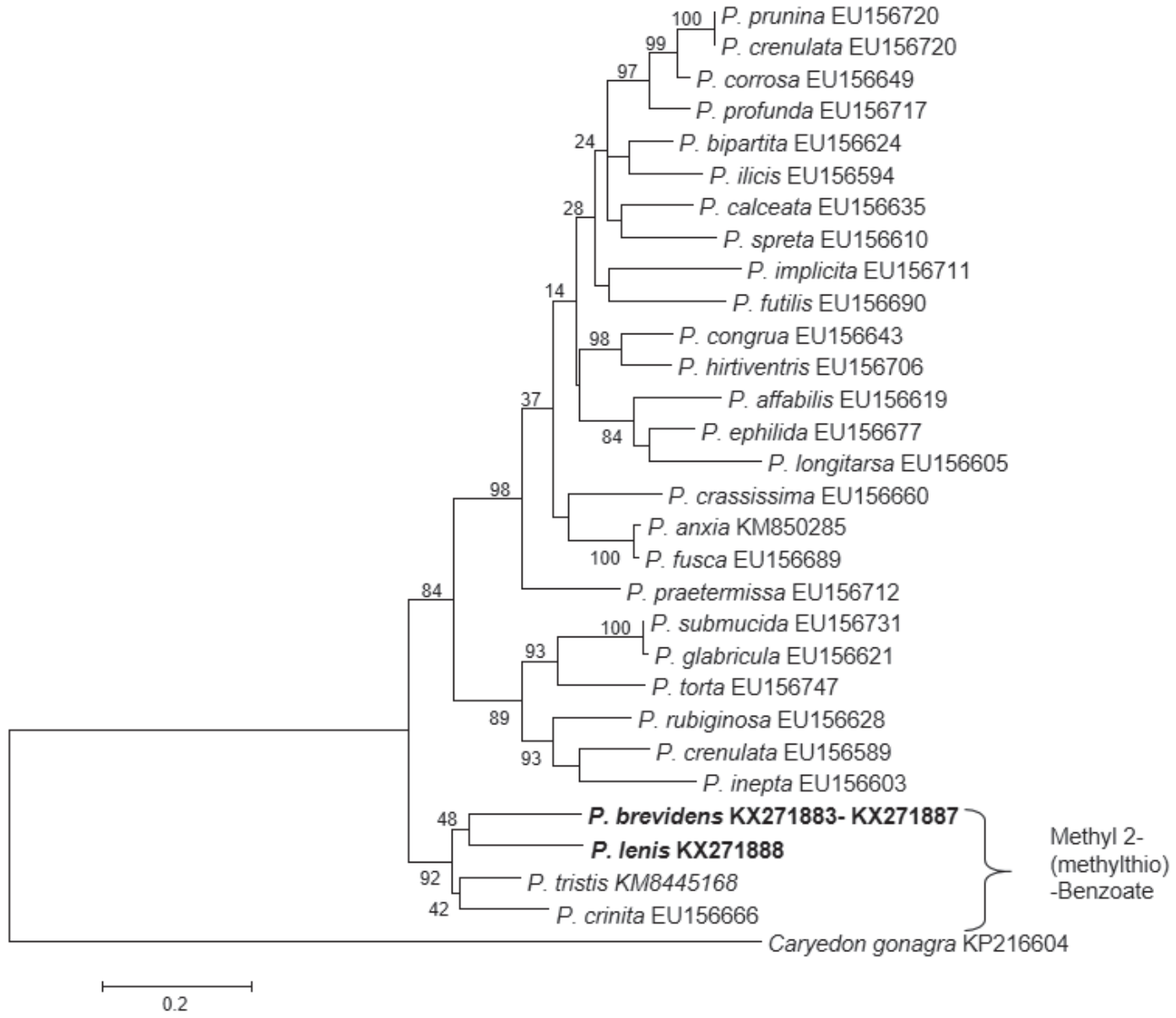

Fig. 2. Phylogenetic tree of *Phyllophaga* species according to the cytochrome oxidase 1 gene, determined using the maximum likelihood method, with the general time reversible with a gamma distribution and invariant sites (GTR + G + I) nucleotide substitution model, and 500 bootstrap repetitions. The species caught in Morelos are shown in boldface.

during those years, so this attractant seems to provide some degree of selectivity and may be useful to reduce the time and labor associated with sorting through trap captures to determine the abundance of *P. brevidens* and *P. lenis*.

Phylogenetic analysis of nucleotide sequences of the mitochondrial *CO1* gene from this study and GenBank showed that the *Phyllophaga* species caught with methyl 2-(methylthio)benzoate are closely related. The sharing of a response to an attractant can help us to understand the evolution and diversification process of the species (Coca-Abia and Robbins 2006; Robbins et al. 2011).

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