Molecular detection method developed to track the koinobiont larval parasitoid *Apanteles opuntiarum* (Hymenoptera: Braconidae) imported from Argentina to control *Cactoblastis cactorum* (Lepidoptera: Pyralidae)

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Abstract

Apanteles opuntiarum Martínez & Berta (Hymenoptera: Braconidae) is a native natural enemy of the cactus moth, *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) in Argentina, where the 2 species are believed to have co-evolved. *Cactoblastis cactorum* is an established invasive pest in the US that is rapidly spreading throughout the southeast. *Apanteles opuntiarum* was imported from Argentina, and reared at the Division of Plant Industry containment facility in Gainesville, Florida, for study as a possible biocontrol agent for release in the US to control *C. cactorum*. A DNA barcode was developed to enable the identification of the reared parasitoid population. The mitochondrial cytochrome oxidase I (*COI*) gene of the *A. opuntiarum* reared in Florida containment was found to be identical to its Argentine founders, but distinctly different from the *COI* sequences of all other reported *Apanteles* species in the NCBI (National Center for Biotechnology Information) GenBank. Additionally, the AoF1 and AoR1 primer pair developed in this study specifically amplified the *COI* gene of *A. opuntiarum*, but did not amplify the *COI* gene of the host *C. cactorum*. Therefore, the *COI* gene fragment identified in this study has the potential to be used as a DNA barcode specific to *A. opuntiarum* that can aid in tracking and identifying this parasitoid inside hosts.

Key Words: biological control; host range tests; *Opuntia*; parasitoids; *COI* gene; DNA barcode

Resumen

Apanteles opuntiarum Martínez & Berta (Hymenoptera: Braconidae) es un enemigo natural de la polilla de la tuna *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) en Argentina, su rango nativo, donde han co-evolucionado. *Cactoblastis cactorum* es una especie invasora establecida en Estados Unidos, que se está dispersando rápidamente hacia el sudeste de este país. *Apanteles opuntiarum* fue importado desde Argentina y es criado en la cuarentena de Gainesville, Florida (Division of Plant Industry), donde está siendo evaluado como posible agente de control de *C. cactorum*, para ser liberado en Estados Unidos. Se desarrolló un código de barras de ADN para permitir la identificación de la población de parasitoides criada. Se encontró que el gen de la citocromo oxidasa mitocondrial I (*COI*) de los *A. opuntiarum* criados en Florida fue idéntico al de sus fundadores argentinos, y claramente diferente de las secuencias de *COI* de todas las demás especies de *Apanteles* reportados en el GenBank del NCBI (Centro Nacional de información sobre biotecnología). Además, el par "primer" AoF1 y AoR1 desarrollado en este estudio amplificó específicamente el gen *COI* de *A. opuntiarum*, y no amplificó el gen de la *COI* del hospedador *C. cactorum*. Por lo tanto, el fragmento del gen *COI* identificado en este estudio tiene el potencial para ser utilizados como un código de barras de ADN específico para *A. opuntiarum* que puede ayudar en el seguimiento y la identificación de este parasitoide dentro de los hospedadores.

Palabras Clave: control biológico; espectro de hospedadores; *Opuntia*; parasitoide; gen *COI*; código de barras de ADN

Opuntia species are used as agricultural and ornamental plants throughout the world because of their ability to adapt to different climatic conditions (Hanselka & Paschal 1989). This genera of cactus is native to the Americas, and is richly represented in the flora of the Western Hemisphere (Anderson 2001). Many *Opuntia* spp. became pests after being introduced and established in different regions of the world. Some of the most dramatic weedy cactus situations occurred in Australia and South Africa, where a classical biological control program using the cactus moth, *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae), brought these weedy outbreaks under control (Dodd 1940; Pettey 1947). *Cactoblastis cactorum* is native to southern South America, primarily Argentina, Uruguay, and Paraguay. However, *C. cactorum*

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was found in Florida in 1989 (Habeck & Bennett 1990; Dickle 1991) and became an invasive insect in North America, feeding on *Opuntia* species and severely damaging the cladodes (Jezorek et al. 2010; Varone et al. 2012). The insect primarily spread along the coasts, and occurs as far north as North Carolina (J. Driscoll, North Carolina Department of Agriculture and Consumer Services, personal communication) and as far west as Louisiana (Hight & Carpenter 2009). *Cactoblastis cactorum* poses a serious threat to native and endangered opuntioid species in the southwestern US, where the plants provide nesting sites and food for a variety of wildlife and contribute to soil stability (Hight & Carpenter 2009). Also, the insect has the potential to become a major infestation of commercial and agricultural cactus crops in Mexico (Zimmermann et al. 2001).

The rapid spread of *C. cactorum* throughout the Atlantic and Gulf coasts of the US requires the development of an immediate control measure (Hight et al. 2002). Aerial application of insecticides has been suggested as a measure to control *C. cactorum* (Vigueras & Portillo 2001; Bloem et al. 2005); however, the use of chemicals is not recommended due to the presence of *Opuntia* species in sensitive ecological areas (Habeck & Bennett 1990, Bloem et al. 2005). Additionally, the aerial spray of insecticides may not be effective because of the endophagous nature of *C. cactorum* (Vigueras & Portillo 2001). Hence, other control measures, including biological control, sanitation by removal of infested cladodes, and sterile insect releases, are appealing as management tools to control *C. cactorum*. Implementation of sanitation efforts coupled with the Sterile Insect Technique (SIT) was used to slow the geographical expansion of *C. cactorum* in the US (Hight et al. 2002; Bloem et al. 2007; Hight & Carpenter 2016); however, SIT was discontinued in 2012 due to the high cost and difficulties associated with applications in swampy environments in Louisiana. The suite of identified natural enemies of *C. cactorum* in Argentina is comprised of at least 8 or 9 parasitoid species (Pemberton & Cordo 2001). However, none of these parasitoids are considered host specific, and are unsuitable for importation and release into the US. *Apanteles alexanderi* Brèthes (Hymenoptera: Braconidae) was identified as a potential biological control agent of *C. cactorum*, but the broad host range of this parasitoid became an issue (De Santis 1967) in pursuing this insect and hindered its use as a biological control agent in North America (Pemberton & Cordo 2001). The recent determination that *A. opuntiarum* had a limited host range in Argentina (Martinez et al. 2012; Goñalons et al. 2014; Varone et al. 2015) made this parasitoid relevant as a potential biological control agent for the control of *C. cactorum*. Female *A. opuntiarum* attack and oviposit eggs inside the larvae of *C. cactorum*. The parasitoid egg hatches and the larva feeds upon the host *C. cactorum* larva until the death of the host. Ultimately, the larval host produces a single brood of parasitoid cocoons in a cluster form (Varone et al. 2015).

Given the importance of *A. opuntiarum* as a potential biological control agent, this parasitoid was imported from Argentina and reared in the containment facility at the Florida Department of Agriculture and Consumer Services (FDACS), Division of Plant Industry (DPI), Gainesville, Florida, USA, to assess the parasitism risk on native North American cactophagous lepidopteran insects. Assessment of the impact of biological control agents on non-target native species is required in the US as a prior condition to their release into the environment to regulate the target species (Van Driesche 2004). Significant challenges during quarantine studies prevented us from obtaining data from the host range tests. In general, the North American native cactus moths are not well studied, and no rearing protocol exists. The native cactophagous species collected in low humidity and hot environments in the southwestern US have been difficult to rear in the humid, air-conditioned, quarantine facility. High mortality was recorded for all native cactus moths collected in the southwestern USA, whether they were or were not challenged with the parasitoid. In the realm of rearing the non-target species, there remains limited concrete progress. The species has proven to be quite fragile to current lab conditions, and survival beyond the larval instar stages ends in failure for most of the southwestern species. This led to the need to distinguish between non-targets that died because of rearing issues (high humidity, cool temperatures, etc.) and non-targets that died because they were attacked by *A. opuntiarum*.

Insects are the largest and most diversified class of invertebrates (Chapman 2006). Some insects are harmful to plants, while some are useful as biological control agents. Although insects from different genera look morphologically different as adults, identification of their larvae can be a difficult task. DNA barcoding has emerged as a molecular tool of importance for the identification of diversity among insect species, and has advantages over more traditional morphological methods (Ratnasingham & Hebert 2007). The mitochondrial cytochrome oxidase subunit I (*COI*) gene lacks introns and does not undergo recombination, which makes it an ideal marker for the identification of insects (Folmer et al. 1994; Hebert et al. 2003a). The objective of this study was to develop a molecular technique that would identify larval *A. opuntiarum* parasitoids inside host insects, either the non-target species or the target species, *C. cactorum*. The *COI* barcode developed in this study was used to identify larvae of *A. opuntiarum* reared inside hosts at Florida Department of Agriculture and Consumer Services, Division of Plant Industry, and could selectively detect *A. opuntiarum* in parasitized *C. cactorum* larvae at the molecular level.

Materials and Methods

DNA EXTRACTION, PCR, AND SANGER SEQUENCING

Apanteles opuntiarum were imported from Argentine field sites inside *C. cactorum* larval hosts (Argentina isolate). A Gainesville culture (GNV) was reared at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry quarantine facility, Gainesville, Florida, USA. Total genomic DNA was extracted from adult *A. opuntiarum*, *A. opuntiarum*-parasitized *C. cactorum* larvae, and non-parasitized larvae of *C. cactorum*, using the DNeasy® Blood and Tissue Kit (QIAGEN, Valencia, California, USA). The quality and quantity of DNA were determined with the NanoDrop derived spectrophotometer A260/A280 nm ratio (2000 Spectrophotometer, NanoDrop Technologies, LLC, Wilmington, Delaware, USA). A pair of universal forward C1-J-1632 (5′-TGATCAAATTTATAAT-3′) and reverse C1-N-2191 (5′-GGTAAAAATTAAAATATAAACTTC-3′) primers were used for *COI* amplification (Kambhampati & Smith 1995) with the GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, California, USA).

The master mix consisted of 5 µL of reaction buffer (2×), 15.5 µL ddH_2O, 1 µL of dNTP, 1 µL of each primer, 0.5 µL KAPA HiFi HotStart DNA Polymerase, and 1 µL template DNA. The final reaction volume of 25 µL was used for the PCR reaction. The PCR protocol included an initial denaturing step at 95 °C for 2 min, followed by 30 cycles of 95 °C for 20 s, 40 °C for 30 s, and 72 °C for 15 s with a final extension at 72 °C for 7 min. The amplified 590-bp DNA fragments were resolved on a 1.2% (w/v) agarose gel (1 × Tris Acetate-EDTA buffer), stained with SYBR Safe dye, and visualized with a UV light imager (Kodak Image Station 4000mm Pro, Kodak, Rochester, New York, USA). The PCR products were cleaned using Roche High Pure PCR Product Purification Kit (Roche Diagnostics Corporation, Indianapolis, Indiana,

USA). The amplified products of *A. opuntiarum* were cloned using the TOPO™-TA Cloning® Kit (Invitrogen, Carlsbad, California, USA) following the manufacturer's instructions. Plasmid DNA was extracted from 5 clones generated from *A. opuntiarum* using the QIAprep® Spin Miniprep Kit (QIAGEN, Valencia, California, USA), and sequencing reactions were carried out using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). Bi-directional Sanger sequencing was performed using the same initial amplification primer with the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA).

DNA SEQUENCE ANALYSIS

Trace files of *COI* DNA sequences obtained from Sanger sequencing were manually verified by analyzing their chromatograms in BioEdit (https://www.mbio.ncsu.edu/BioEdit/bioedit.html). DNA sequences at the 5′ and 3′ ends were trimmed and submitted to the NCBI GenBank (accession numbers KY986878 and KY986879). Multiple alignments of *COI* gene sequences were performed using the Clustal W program (www2.ebi.ac.uk/clustalw), and phylogenetic analyses were conducted using MEGA 7 (Kumar et al. 2016). A phylogenetic consensus tree of *COI* sequences was developed using the Neighbor-Joining method with bootstrap values of 1,000 replicates (Kumar et al 2016).

SPECIES-SPECIFIC PRIMER DESIGN

The *COI* sequences of *A*. *opuntiarum* (JX566778), *A*. *alexanderi* (JX566789), *Apanteles* sp. Rodriguez (HQ926431), *Apanteles* sp. Janzen15 (KF462200), *Apanteles fumiferanae* Viereck (JF864061), *Apanteles morrisi* Mason (KR789179), and *Apanteles polychrosidis* Viereck (HQ558926), (all Hymenoptera: Braconidae) were retrieved from the NCBI GenBank database (http://www.ncbi.nim.nih.gov/), and were aligned using Clustal W (Thompson et al. 1994). Species specific primer was designed visually in the gene region where there was a mismatch pair related with other species. A final pair of *A*. *opuntiarum*-specific primers were designed for the detection of *A*. *opuntiarum* parasitoids: forward primer AoF1 (5′-AGGATTTATTAACACAGGAGTTGGGAC-3′), and reverse primer AoR1 (5′-TGGGTCACCACCACCAG-3′) (Fig. 1).

VALIDATION OF SPECIES-SPECIFIC PRIMERS

Primer specificity was validated by PCR amplification of the *COI* gene region using extracted DNA from parasitized and non-parasitized target host larvae (*C. cactorum*), and other non-target hosts (Table 1). The ReadyMix PCR Kit, KAPA HiFi HotStart DNA Polymerase (KAPA Biosystems, Wilmington, Massachusetts, USA) was used for amplification. The final reaction volume of 25 µL consisted of 5 µL of reaction buffer (2×), 15.5 µL ddH_2O, 1 µL of dNTP, 1 µL of each primer, 0.5 µL KAPA HiFi HotStart DNA Polymerase, and 1 µL template DNA. The PCR cycler conditions used were an initial denaturation at 95 °C for 2 min, followed by 30 cycles of 98 °C for 20 s, 52 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min. The amplified 340-bp DNA fragments were resolved on a 1.5% (w/v) agarose gel (1 × Tris Acetate-EDTA buffer), stained with SYBR Safe dye, visualized with a UV light imager (Kodak Image Station 4000mm Pro, Kodak, Rochester, New York, USA), and were sequenced in both directions with the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). In each PCR, the positive (*A. opuntiarum*) and negative (water and non-parasitized larvae of *C*. *cactorum*) controls were processed simultaneously with the tested samples to identify possible false-positive results and contamination.

SENSITIVITY TEST OF THE SPECIES-SPECIFIC PRIMERS

Sensitivity testing with the *A. opuntiarum* species-specific primers was determined in PCR amplification with a series of dilutions using decreasing DNA concentrations with the same primer concentration. The DNA concentrations used were 100 ng, 10 ng, 1 ng, 100 pg, and 10 pg, along with a negative control (water). PCR amplification using the species-specific primers in a total reaction volume of 25 µL was performed following the method described above.

Results

PHYLOGENETIC ANALYSIS OF *APANTELES OPUNTIARUM*

The *COI* sequence of *A*. *opuntiarum* was amplified from genomic DNA, and confirmed by Sanger sequencing and nucleotide BLAST analyses. The *COI* gene sequence of *A. opuntiarum* (JX566778) and its homologous sequences from another 9 Braconidae, *A. alexanderi* (JX566789), *Apanteles* sp. Rodriguez (HQ926431), *Apanteles* sp. Janzen15 (HF462200), *A. fumiferanae* (JF864061), *A. morrisi*, *A. polychrosidis*, *Microgastrinae* gen. (KF462133), *Cotesia vestalis* (Haliday) (KY832157), and *Cotesia plutellae* (Kurdjumov) (AY934818), and the host, *C. cactorum* (HQ924004), were obtained from the NCBI GenBank database and were used to generate a phylogenetic tree. The final 557-bp *COI* nucleotide sequence clearly separated the *Apanteles* species into 2 groups, based on phylogeny (Fig. 2). As expected, the *A. opuntiarum* procured from Argentina resembled the *A. opuntiarum* reared at Florida Department of Agriculture and Consumer Services, Division of Plant Industry, and formed a single group along with the earlier reported NCBI GenBank *A. opuntiarum* (JX566778) (Fig. 2). However, other *Apanteles* populations, *Apanteles* sp. Rodriguez (HQ926431), *Apanteles* sp. Janzen15 (HF462200), *A. fumiferanae* (JF864061), *A. morrisi* (KR789179), *A. polychrosidis* (HQ558926), and *Microgastrinae* gen. (KF462133), clearly diverged from the *A*. *opuntiarum* and formed a separate group (Fig. 2). *Cactoblastis cactorum* (HQ924004), *C. vestalis* (KY832157), and *C. plutellae* (AY934818) were evidently separated from *A. opuntiarum* as outgroups (Fig. 2).

MOLECULAR MARKER FOR *APANTELES OPUNTIARUM*

Differences in the *COI* nucleotide sequences of *A*. *opuntiarum*, *Apanteles* sp. Rodriguez (HQ926431), and *Apanteles* sp. Janzen15 (HF462200) each were considered for designing primers to selectively amplify the *COI* of *A*. *opuntiarum*. The designed primers amplified 340-bp amplicons from only *A. opuntiarum* (Gainesville culture), and the parasitized *C*. *cactorum* larvae (Fig. 3a, b). However, *A. alexanderi* and non-target species (Fig. 3a, sample #4) did not show amplification with similar primer and PCR conditions. PCR amplification using DNA from non-parasitized *C. cactorum* also showed no amplification, resulting in a negative control for the designed primers. A DNA band of size 340-bp was amplified using *COI*-specific AoF1 and AoR1 primer pair (Fig. 3A, B). This was observed in both the original Argentina isolate and in the Gainesville culture (Fig. 3A, B). The absence of the 340-bp DNA band (Fig. 3B) in 9 larval specimens of *C*. *cactorum* (specimens 4, 5, 7–9, 11–13, 15) indicated that the primer pair AoF1 and AoR1 selectively amplified the *COI* gene from *A*. *opuntiarum*, but not from the *C. cactorum*. A phylogenetic analysis was performed using the 340-bp fragment (phylogenetic tree not shown); the tree generated was found to be identical to the phylogenetic tree generated earlier using 557-bp. This analysis suggested that the AoF1 and AoR1 primers are valid for use as a DNA barcode for the identification of the *A*. *opuntiarum* life stage inside the *C. cactorum* host larvae.

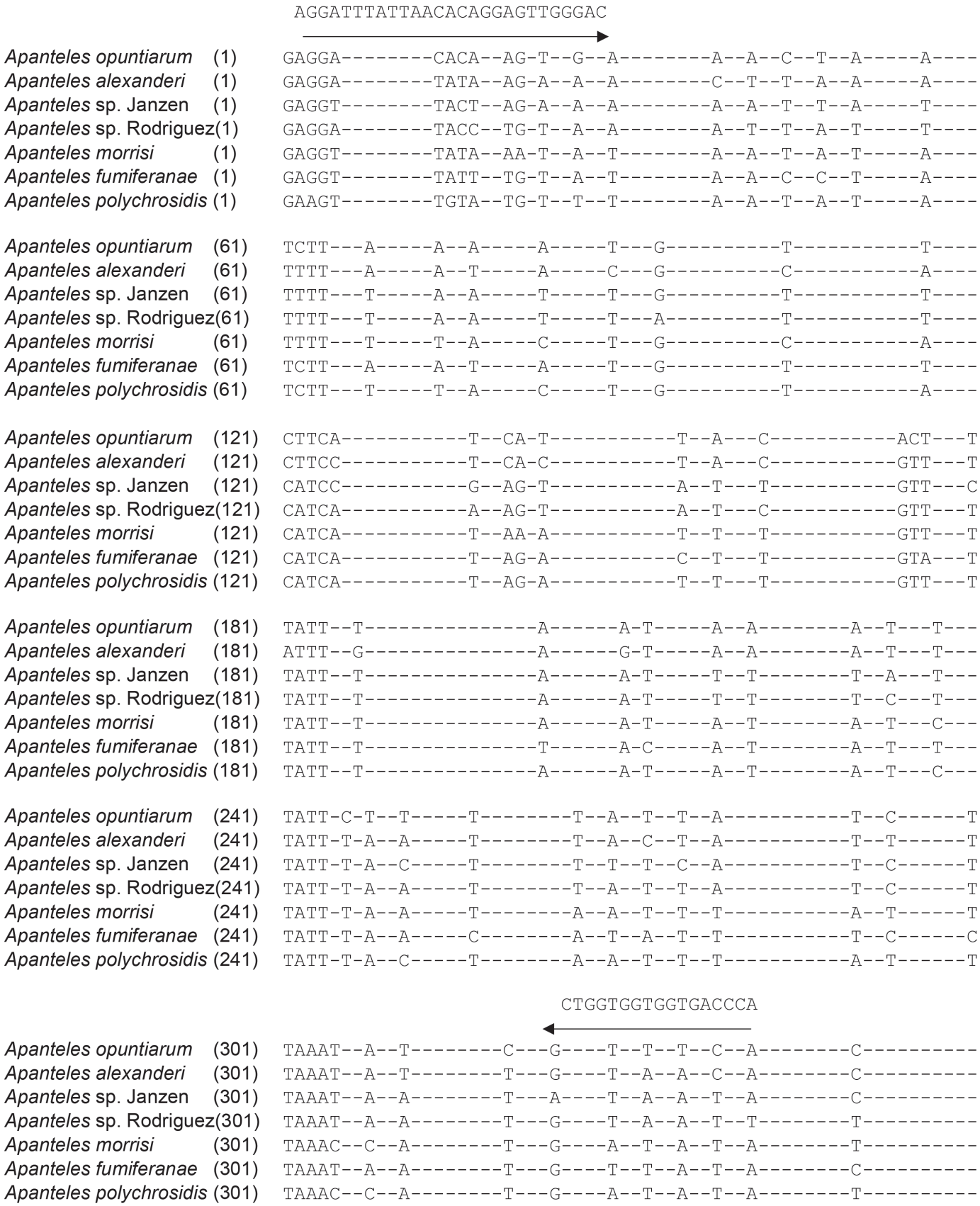

Fig. 1. Primers designed from the sequences of the *COI* gene of *Apanteles* species used for specific amplification and detection of *A. opuntiarum.*

Table 1. List of non-target Lepidoptera species and their collection location which were tested for validation of *Apanteles opuntiarum* primers.

Species	Location
Cactoblastis doddi	Mendoza, Argentina
Melitara prodenialis	Citra, Florida, USA
Melitara prodenialis	USDA-ARS, Tallahassee, Florida, USA
Melitara doddalis	White Sands, New Mexico, USA
Melitara dentata	Safford, Arizona, USA
Melitara dentata	Alpine, Texas, USA
Melitara dentata	Brewster County, Texas, USA

DETECTION OF *APANTELES OPUNTIARUM* USING A DNA BARCODE

Sanger sequencing was used to verify the molecular identity of the 340-bp fragment amplified with the AoF1 and AoR1 primers. The 340-bp *COI* sequences obtained from the parasitoid infested *C. cactorum* and reared *A. opuntiarum* were aligned and compared. The *COI* sequences of parasitoid (*A. opuntiarum*) infested *C. cactorum* were identical to the *COI* sequences derived from the reared *A. opuntiarum*. The presence of an *A. opuntiarum*-specific *COI* sequence in the parasitized larvae of *C. cactorum* indicates that we can track this parasitoid wasp in this host and study the establishment of this parasitoid after its release into the environment.

Sensitivity of the specific primer designed for *A. opuntiarum* was determined using serial dilutions of the *A. opuntiarum* sample. In this species, DNA concentrations of 100 ng, 10 ng, 1 ng, 100 pg, and 10 pg resulted in strong intensity bands and suggested the variable range of template DNA for detection of *A. opuntiarum* (Fig. 4).

Discussion

DNA barcoding is a significant tool that allows for the identification of organisms at the molecular level (Gariepy et al. 2014), while molecular tracking is a useful tool to investigate the release and establishment of parasitoids in the field (Persad et al. 2004; Gariepy et al. 2014). This approach allows for the detection of both the host and the parasitoid within 1 sample, which makes for a fast and cost-effective identification method. The *COI* was reported as a DNA barcode to track the management of arthropods and their parasitoids (Gariepy et al. 2007, 2014), and for species identification (Liu et al. 2011). Traugott and Symondson (2008) reported detection of parasitoid genetic material in *Aphis fabae* Scopoli (Hemiptera: Aphididae) immediately after the oviposition by the female parasitoid. The *COI* also was reported as a genetic marker in various animal groups and metazoan invertebrates (Folmer et al. 1994, Lunt et al. 1996, Hebert et al. 2003b). A DNA barcode also was reported for a few genera of parasitoids such as *Diachasmimorpha* (Jones et al. 2005), *Psyttalia* (Rugman-Jones et al. 2009), and *Lysiphlebia* (Zhou et al. 2014) (all Hymenoptera: Braconidae).

In this study, we developed a DNA barcode to detect *A. opuntiarum* within an individual cactophagous larva in the early stages of development. The *COI* gene sequence was amplified from the parasitoid *A. opuntiarum* to confirm the identity of the reared *A. opuntiarum* and its specific host, *C. cactorum*, as the control. Analysis of the *COI* gene fragment from *A. opuntiarum* reared at the quarantine facility at Florida Department of Agriculture and Consumer Services, Division of Plant Industry revealed its identity with an earlier reported *COI* gene (GenBank accession number JX566778) from *A. opuntiarum.* The *COI* genes of different *Apanteles* species obtained from the NCBI GenBank database showed significant differences among each other, and formed 2 sepa-

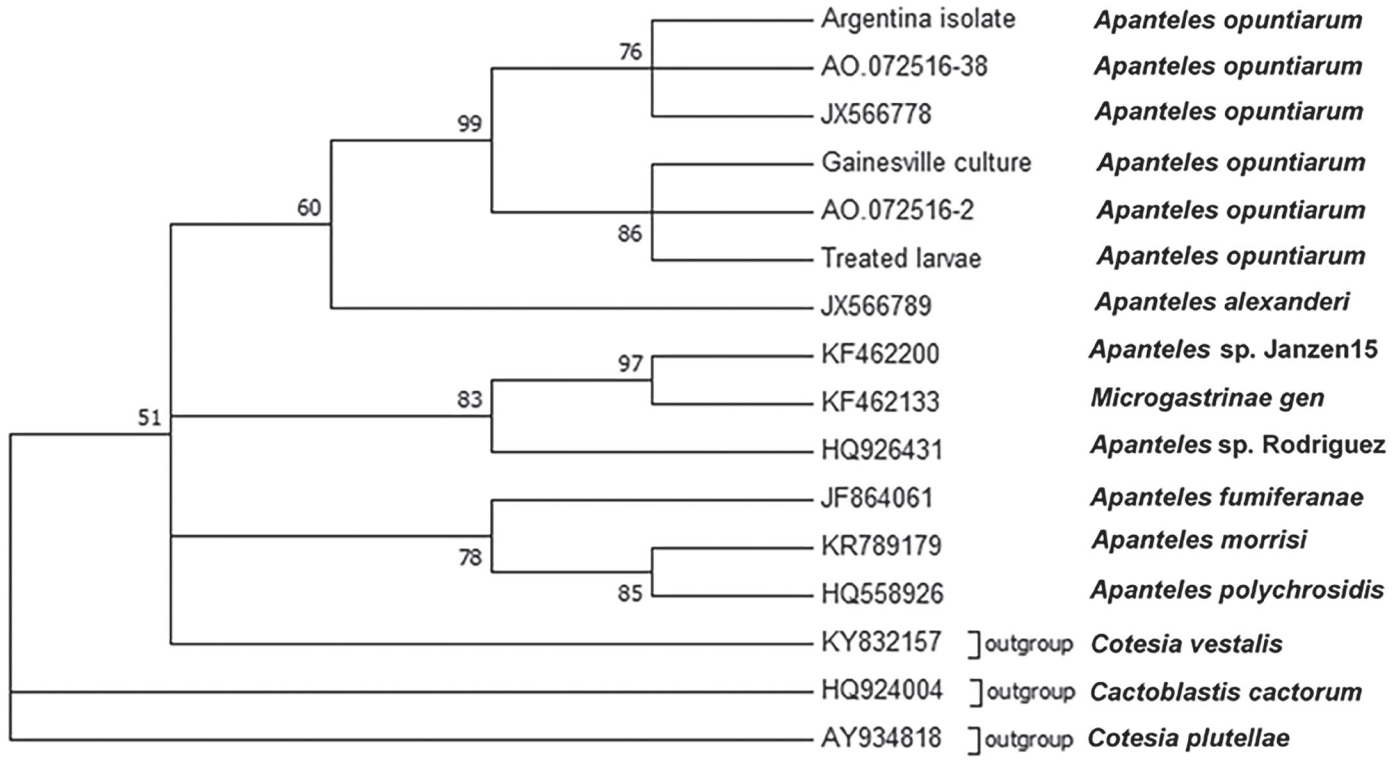

Fig. 2. Phylogenetic tree of *Apanteles* species. Argentina isolate (*A. opuntiarum* collected in Argentina), Gainesville culture (*A. opuntiarum* obtained from Argentina and reared at quarantine facility in Gainesville, Florida, USA), JX566778 (*A. opuntiarum*), JX566789 (*A. alexanderi*), HQ926431 (*Apanteles* sp. Rodriguez), and KF462200 (*Apanteles* sp. Janzen 15). Phylogenetic tree of *COI* nucleotide sequences were subjected to bootstrap test with 1,000 replicates using MEGA 7. Numbers on the branch lengths are bootstrap values.

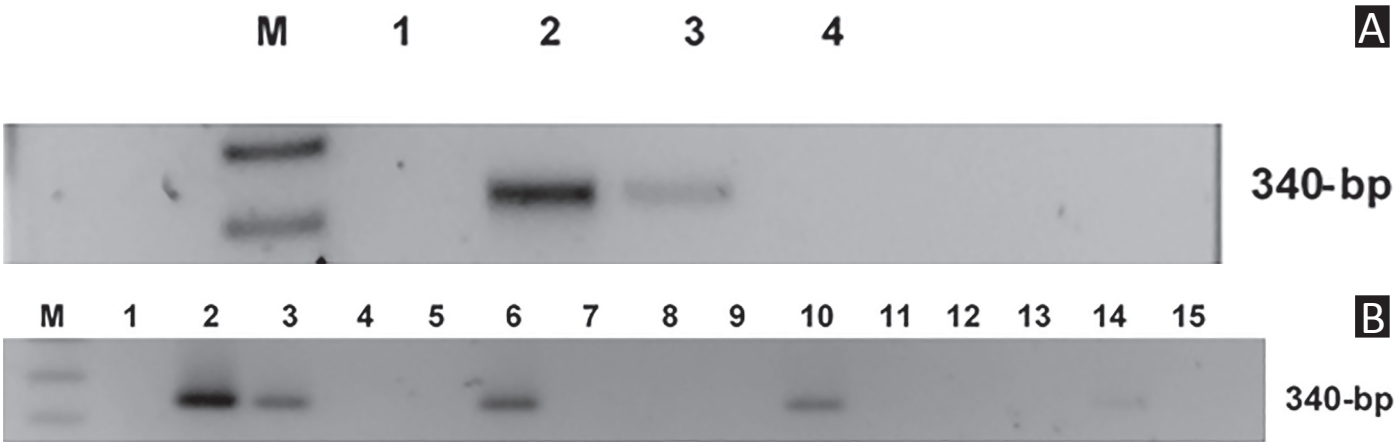

Fig. 3. (A) Gel electrophoresis showing PCR based identification of *Apanteles opuntiarum*. M: DNA marker (exACTGene low range Plus DNA Ladder); 1: negative control without template DNA; 2: positive control Gainesville culture (*A. opuntiarum* reared at quarantine facility, Gainesville, Florida, USA); 3: parasitized *Cactoblastis cactorum* larvae; 4: unparasitized *C. cactorum* larvae. (B) Gel electrophoresis showing PCR based identification of *Apanteles opuntiarum*. M: DNA marker (exACTGene low range Plus DNA Ladder); 1: negative control without template DNA; 2: Argentina isolate (*A. opuntiarum* from Argentina); 3: Gainesville culture (*A. opuntiarum* reared at quarantine facility, Gainesville, Florida, USA); 4 to 15: *Cactoblastis cactorum* larvae exposed to *A. opuntiarum*.

rate groups in a phylogenetic tree. Variation in the *COI* gene sequences among *Apanteles* species led to the identification of a DNA barcode to specifically amplify the *COI* gene of the parasitoid *A. opuntiarum*. The molecular technique is sensitive to oviposition rather than a sting, because stinging releases only protein, but oviposition releases an egg(s) that contains nucleic acids. Developing a molecular marker is not an expensive technique, and can be accomplished faster with greater accuracy than the conventional technique of rearing test individuals. The molecular method detects only the presence of parasitoid nucleic acids (DNA and RNA), therefore, the detection of parasitoid eggs in host larvae is carried out with high selectivity and rapidity. However, the molecular method does not indicate if the parasitoid could complete its life cycle in the host. PCR analysis only checks whether a parasitoid attacks and oviposits into the host; it cannot reveal whether the oviposited eggs are developing or not. However, this method has been used successfully in our quarantine host range tests. Based on our results of identifying parasitized hosts by genetic techniques, we found this method promising and helpful in concluding the host specificity of *A. opuntiarum.*

Therefore, the *COI* barcode identified in this study can be used for the identification and management of *A. opuntiarum*. Furthermore, the parasitic nature of *A. opuntiarum* was confirmed when DNA obtained from parasitized larvae of *C. cactorum* showed the presence of *A. opuntiarum* in the parasitized host larvae. Non-target species from the southwestern US are quite fragile, and their survival beyond the larval instar stages often ends in failure for most of the species. The molecular technique developed in this study has been useful to perform host range studies, and to evaluate the ability of *A. opuntiarum* to parasitize non-target host species, if any exist. In comparison to the time and resource consuming traditional rearing method for detection of parasitism, the molecular technique is considerably less labor intensive and relatively easy to perform. Therefore, this DNA barcode will be essential for the identification of *A. opuntiarum*, and can later be used to track the parasitoids' establishment when released into the field for biological control.

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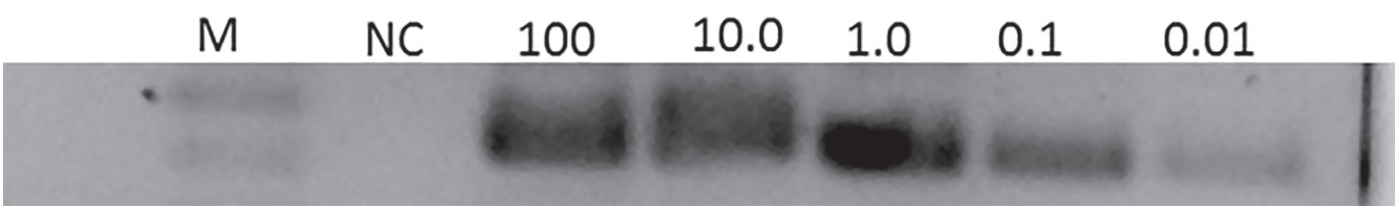

Fig. 4. Gel electrophoresis showing PCR based sensitivity testing of *Apanteles opuntiarum* with a series of samples using decreasing DNA concentrations with the same primer concentration. The DNA concentrations used were 100, 10, 1, 0.1, 0.01 ng µL−1; M: DNA marker (exACTGene low range Plus DNA Ladder); NC: Negative Control without template DNA.

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