Isolation and DNA Barcode Characterization of a Permanent *Telenomus* (Hymenoptera: Platygastridae) Population in Florida That Targets Fall Armyworm (Lepidoptera: Noctuidae)

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ABSTRACT *Telenomus remus* Nixon is a platygastrid egg parasite of the fall armyworm, *Spodoptera frugiperda* (J. E. Smith), with a history of use as an augmentative biological control agent in Central and South America. Efforts were made in 1975–1977 and again in 1988–1989 to introduce *T. remus* into the fall armyworm overwintering regions of southern Florida to mitigate infestations by this migratory pest, but in neither case was evidence of long-term establishment found. However, in 2009 and again in 2013, an unidentified *Telenomus* species was found attacking fall armyworm sentinel egg masses placed on corn plants or pasture grasses in the north-central Florida counties of Levy and Alachua. Taxonomic uncertainties have so far not allowed a conclusive identification of species by morphological keys. DNA barcode comparisons showed a single Florida haplotype in all collections that was identical to that found in a *T. remus* colony from Ecuador and very similar to a *T. remus* colony from Honduras. The *T. remus* barcode sequences were phylogenetically distinct from a second *Telenomus* species from Ecuador, *T. rowani*, and from other related sequences obtained from the NCBI GenBank database. This represents the first observation of a permanent *Telenomus* population in the United States that targets fall armyworm and provides genetic evidence for its identification as *T. remus*. These findings have positive implications for the use of augmentative biological control methods to mitigate fall armyworm migration from Florida.

KEY WORDS *Spodoptera frugiperda*, parasitoid, biological control

Telenomus remus Nixon (Hymenoptera: Platygastridae) is a lepidopteran egg parasitoid that is currently being mass reared and released in several Central and South American countries for the control of *Spodoptera* (Noctuidae) pest species, in particular the fall armyworm *Spodoptera frugiperda* (J. E. Smith) (Cave 2000). This small parasitic wasp is noteworthy for its aggressive and efficient ability to parasitize the large egg masses associated with fall armyworm, whose multiple, superimposed layers are covered with scales that limit attack from other parasitoids (Cave and Acosta 1999). *T. remus* can penetrate all layers of the egg mass, routinely producing 80–100% parasitization in laboratory studies (Wojcik et al. 1976, Pomari et al. 2012), characteristics that favor its use as a biological control agent. *T. remus* is a native of Asia and has been introduced into the Western Hemisphere in multiple locations and times with successful establishment demonstrated in parts of the Caribbean, Venezuela, and Honduras (reviewed in Cave 2000), and evidence found for permanent populations in Ecuador (Pomari et al. 2012). Augmentative releases of *T. remus* and other *Telenomus* species continue to be used in biological control programs in Central and South America, so the geographical distribution of these species in this region is likely to be both variable and expanding (Figueiredo et al. 2002, Van Lenteren and Bueno 2003, Gutiérrez-Martínez et al. 2012).

Cave (2000) discussed the difficulty of distinguishing *T. remus* from native species that have overlapping host range. Specifically, *Telenomus minutissimus* Ashmead is native to the Caribbean and can parasitize *Spodoptera* spp., as does *Telenomus solitus* Johnson, a native of Guatemala and Honduras (Yaseen et al. 1981, Cave 2000). Both species have similar external morphologies with *T. remus*, including the male genitalia (Cave 2000). In addition, *Telenomus nawai* Ashmead and *Telenomus spodopterae* (Dodd) are Eastern Hemisphere species with similar host ranges as *T. remus*, with *T. nawai* having been released in the Caribbean in 1980 (Braithwaite and Pollard 1981). It has been suggested that *T. nawai*, *T. spodopterae*, and *T. minutissimus* are synonymous species with *T. remus*, though this has not been established (Braithwaite and Pollard

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1981, Yaseen et al. 1981, Centre for Agriculture Bioscience International (CABI) 2014). These taxonomic uncertainties and morphological ambiguities complicate attempts to identify and characterize the populations of *T. remus* and closely related species in the Western Hemisphere.

Fall armyworm is the primary pest of sweet corn and forage corn in Florida and is an important pest of corn, sorghum, and turf grasses in the United States, with occasional breakout years of more intensive infestations (Luginbill 1928, Sparks 1979). The pest can be found throughout the central and eastern United States, extending as far north as Canada, but is only able to overwinter in southern Florida and Texas (Pair and Sparks 1986, Nagoshi et al. 2012). It has long been thought that controlling the overwintering populations with natural enemies, such as *T. remus*, could be an effective means of mitigating the migratory infestations (Wojcik et al. 1976, Knipling 1980, Waddill and Whitcomb 1982). With this objective in mind, there was an attempt in 1975–1977 to establish *T. remus* in the overwintering regions of Florida with the release of over 660,000 adults in the Homestead area just south of the city of Miami (Waddill and Whitcomb 1982). These wasps were mass reared in Trinidad and originated from populations established in Barbados that originated from Malaysia (Waddill and Whitcomb 1982, Cave 2000). A second mass release in the same area was performed in 1988–1989 with approximately 110,000 adults that originated from established populations in Puerto Rico and the Cayman Islands (Bennett 1994, Releases of Beneficial Organisms in the United States and Territories [ROBO] 2008). In neither case were parasitoids recovered after termination of the releases, indicating that the establishment of a large population was unsuccessful.

This paper describes the existence since 2009 of a *Telenomus* population in north-central Florida that uses fall armyworm as a host, and provides genetic evidence that it is likely to be *T. remus*. DNA barcoding methods were used to better characterize the Florida population and its relationship with *Telenomus* populations in Central and South America (Hebert et al. 2003). In this technique, the DNA sequence of a portion of the mitochondrial *Cytochrome Oxidase Subunit I* gene (*COI*) was compared among different populations. The degree of sequence similarity typically relates to the taxonomic relatedness of the populations, which can be measured by phylogenetic analysis. Specifically, we used DNA barcoding to compare *T. remus* from Honduras and Ecuador with each other and with the *Telenomus* species identified in Florida. We also compared all populations with *Telenomus rowani* (Gahan) from Ecuador, to assess the ability of DNA barcoding to discriminate between sibling *Telenomus* species.

Materials and Methods

Source of *Telenomus* Specimens from Ecuador and Honduras. Specimens of *T. remus* and *T. rowani* were obtained from laboratory colonies maintained at the Instituto Nacional Autonomo de Investigaciones Agropecuarias, Guayas, Ecuador. The length of time in colony and frequency of new field infusions are uncertain. Fall armyworm eggs from a laboratory colony were placed in rice fields where they were vulnerable to infestation by parasitoids like *Telenomus*. Similarly, *T. rowani* were collected using sentinel eggs from a laboratory colony of the rice borer *Rupela albinella* (Cramer) placed in rice fields. Colonies were maintained on fall armyworm or *R. albinella* eggs with periodic supplementation with field-collected specimens. *Telenomus remus* from Honduras was obtained from laboratory colonies maintained at the Escuela Agricola Panamericana, Zamorano, Tegucigalpa, Honduras.

Collecting *Telenomus* in Florida. *Telenomus* specimens were collected in the spring of 2009 using sentinel egg masses as part of a multi-year study on fall armyworm natural enemies in Florida. Fall armyworm egg masses were obtained from colonies maintained at the USDA-ARS, CMAVE Behavior and Biocontrol Unit in Gainesville, FL. Females were allowed to oviposit on paper towels the night before the experiment. Paper segments (25 by 25 mm) containing the eggs were pinned to the underside of leaves in pastures at a cattle farm in Levy County, FL (Table 1). The eggs were left in the field for 36–48 h then brought to the laboratory for further rearing in 55 by 15 mm Petri dishes. Emerging fall armyworm larvae were promptly removed to avoid egg damage. A subset of the adult parasitoids that emerged from these field-collected eggs was preserved in 70% ethanol and stored until taxonomic identification and DNA analysis. The remaining parasitoids were used to establish a laboratory colony using the following procedure. The parasitoids were maintained in a clear, round, wide mouth plastic containers with screw caps that were 13.5 (d) by 14 (h) cm. The containers were placed upside down so that the bottom of the container was kept facing upward. A circular 6-cm screen was placed on the bottoms of the containers for ventilation and the screw lid was used when access to the colony was required. Several smears of honey were placed on the interior walls of the containers and pieces of paper towel saturated with water were embedded in a 30-ml acrylic Solo cup (Solo Cup Co., Urbana, IL) for parasitoid feeding. The parasitoids were allowed to mate for 3 d and then masses of fall armyworm eggs were offered for oviposition. All rearing was done at 24°C, 65% humidity, and with a photoperiod of 14:10 (L:D) h. Specimens for DNA analysis were collected from the colony in 2013 and placed in 70% ethanol.

Telenomus was again collected using the same method in July 2013 in cornfields at the University of Florida Dairy Research Unit in Alachua County, FL. Adult parasitoids were stored in 70% ethanol prior to DNA isolation and analysis. Voucher specimens are stored at the USDA-ARS, CMAVE Behavior and Biocontrol Unit in Gainesville, FL.

DNA Extraction and PCR Amplification. *Telenomus* specimens are very small (length of 1 mm) and so present special challenges for

Table 1. *Telenomus* collection and DNA sequence information

Source Location	Source type	Collection date	Species[a]	*n*	DNA sequence[b] PCR1	PCR2	FAW	Failed
Levy Co., FL	Field	Oct. 2009	Unknown	10	3	0	5	2
Levy Co., FL	Colony	Oct. 2009	Unknown	9	4	4	0	1
Yaguachi, Ecuador	Colony	June 2013	*T. remus*	14	3	4	3	4
Tegucigalpa, Honduras	Colony	June 2013	*T. remus*	26	15	5	0	6
Yaguachi, Ecuador	Colony	June 2013	*T. rowani*	14	6	6	0	2
Alachua Co., FL	Field	July 2013	Unknown	42	33	5	0	4
			Total:	115	64	24	8	19

[a] Taxonomic identification based on morphology.
[b] PCR1: *Telenomus* DNA sequence data obtained from first PCR amplification. PCR2: *Telenomus* DNA sequence data required re-amplification of the first PCR reaction. FAW: Number of specimens identical to fall armyworm *COI*. Failed: No sequence was obtained.

DNA isolation. Individual specimens were placed in 0.8 ml of DNA homogenization buffer (Zymo Research, Orange, CA) and homogenized in a 5-ml glass Potter homogenizer (Bellco Glass, Inc, Vineland, NJ). Homogenate was added directly to a Zymo-Spin I column (Zymo Research, Orange, CA) and processed according to manufacturer's instructions. Purified DNA was eluted to a final volume of 10 µl with double distilled water. PCR amplification was first performed on four specimens from the *Telenomus* colony with primers COI-58 F (5′-GGAATTTGAGCAGGAATAGTAGG-3′) and COI-945dR (5′-AATW[A/T]CCTGTTGGTACD [G/A/T]GCA-3′) that amplify the barcode region of the fall armyworm *COI* gene (Nagoshi et al. 2012). PCR amplification was performed using the entire DNA preparation (10 µl). The reaction mix included 3 µl 10X manufacturer's reaction buffer, 0.5 µl 10 mM dNTP, 0.5 µl 20 µM primer mix, 0.5 unit Taq DNA polymerase (New England Biolabs, Beverly, MA), 10 µl sample DNA, in a 30 µl final volume. The thermocycling program was 94°C (1 min), followed by 32 cycles of 92°C (30 s), 52°C (30 s), 72°C (40 s), and a final segment of 72°C for 3 min. The amplified product was gel-purified by the addition of 5 µl of 6X gel loading buffer to each sample, which was then run on a 1.8% agarose horizontal gel containing GelRed (per manufacturer's instructions, Biotium, Hayward, CA) in 0.5X Tris-borate buffer (TBE, 45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0). Fragments were visualized on a long-wave UV light box. Fragment isolation was performed using Zymo-Spin I columns (Zymo Research, Orange, CA) according to manufacturer's instructions. The purified fragments were analyzed by DNA sequence analysis. All four specimens produced the same DNA sequence, which was used to generate the following primers: Tel19Fd (5′-ATTGGAR[G/A]ATGAY[C/T]CAAATTTATAA-3′), TelKF235F (5′-TCAG GAACAGGAACAGGATGAACTGT-3′), and Tel733Rd (5′-GATGW[A/T]GCTCAW[A/T]ACAATAAATCCTA-3′). The degeneracies in Tel19Fd and Tel733Rd were based on comparisons with the fall armyworm *COI* sequence and were incorporated to facilitate amplification of other *Telenomus* species that might be present.

DNA comparisons, alignments, and primer construction were performed using Geneious Pro 6.1.8 (Biomatters Ltd, Auckland, New Zealand). DNA sequencing was performed by the University of Florida Interdisciplinary Center for Biotechnology Research (Gainesville, FL). All primers used for PCR amplifications and DNA sequencing were obtained from Integrated DNA Technologies (Coralville, IA).

Analysis of *Telenomus* Specimens. PCR amplification using primers Tel19Fd and Tel733Rd was performed as described above except that an annealing temperature of 56°C was used. After amplification, 5 µl of the reaction was diluted to 200 µl in water and stored at −20°C for re-amplifications if needed. The remainder was analyzed on a 1.8% agarose gel. Of the 115 samples tested, 64 produced sufficient product after the first PCR reaction (PCR1) for DNA sequence analysis of the gel-purified fragment (Table 1). On the assumption that the remaining 51 samples failed because of low template concentration, they were reanalyzed by two PCR re-amplifications (PCR2) of the diluted initial PCR reaction. One reaction again used Tel19FD/Tel733Rd and the other Tel235F/Tel733Rd (Fig. 1). In each case, 2 µl of the diluted first reaction was amplified using the same reaction mix and conditions described as before. Of these 51, 32 produced PCR products using both primer combinations that could be detected by gel electrophoresis and gave useable DNA sequencing information (Table 1). In every case, the sequences from the two PCR amplifications were identical in the region of overlap.

Descriptive DNA sequence statistics and calculations of nucleotide variation based on the Jukes-Cantor (Jukes and Cantor 1969) model were performed using DNAsp Ver. 5.1 (Rozas and Rozas 1997). Sequence divergences among individuals were calculated within the Geneious Pro 6.1.8 program, again using the Jukes-Cantor distance model and graphically displayed in a neighbor-joining (NJ) cladogram (Saitou and Nei 1987). Confidence was assessed by bootstrapping at 5,000 replications using the *S. frugiperda COI* sequence as outgroup (GenBank EU141360). All haplotypes obtained in this study have been deposited in GenBank (KM485690–KM485699). Voucher specimens are deposited at CMAVE (Gainesville, FL).

Results

DNA sequence information was obtained and compared for a 608-bp portion of the *COI* gene that overlaps a region frequently used for barcode analysis

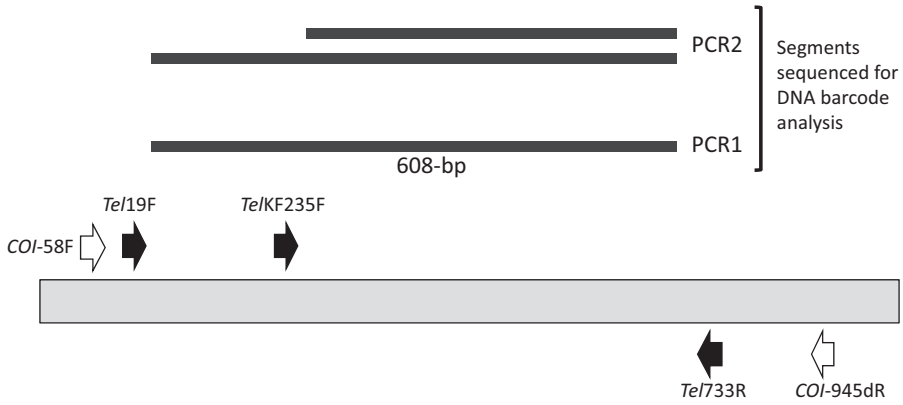

Fig. 1. Diagram of the *COI* region used to perform DNA barcode analysis of the *Telenomus* specimens. Arrows indicate location and direction of primers used for the PCR and DNA sequencing. The *COI*-58F and *COI*-945dR were derived from fall armyworm sequence data and used to obtain the initial *Telenomus* barcode sequence. The 608-bp segment used for the *Telenomus* barcode analysis is indicated as PCR1. The PCR2 analysis required sequencing of the same 608-bp segment and an overlapping 392-bp segment.

(Fig. 1, Hebert et al. 2003). A total of 115 individuals from six *Telenomus* collections were examined, representing four laboratory colonies and two field populations (Table 1). Useable DNA sequence data were obtained from 96 samples of which eight were identical to the *COI* gene from fall armyworm, the developmental host for those specimens. The remaining 86 samples showed highest DNA sequence identity to the *COI* genes belonging to *Telenomus* or Hymenoptera species that were obtained from an NCBI Blast search of the GenBank nucleotide database.

The barcode regions of the *T. remus* specimens from Honduras were compared to those of the *T. remus* and *T. rowani* specimens from Ecuador. Little genetic variability was observed within the two *T. remus* collections, as all seven specimens from Ecuador were identical (haplotype Treh1), as were the 20 specimens from Honduras (Treh2). Treh1 differed from Treh2 at three sites (215, 476, and 596) with none of the polymorphisms altering the predicted amino acid sequence (Fig. 2, Table 2). Substantially higher variation was observed in the *T. rowani* collection, with seven haplotypes produced from 48 polymorphic sites defined by a comparison of 12 specimens (Table 2, Fig. 2).

The same *COI* region was analyzed for the Florida specimens. All eight barcode sequences from the laboratory colony were identical to each other and to the 41 *Telenomus* sequences obtained from field-collected egg masses. The single Florida *COI* haplotype was identical to the Treh1 haplotype associated with the Ecuador *T. remus* colony (Fig. 2). The Treh1 and Treh2 sequences were each used for a BLAST search of the NCBI database. In both cases, greater than 97% sequence identity was found for an unidentified *Telenomus* sp. (GenBank JX559778) and an unidentified Hymenoptera sp. (GenBank KF365460; Fig. 2).

Additonal BLAST searches using the two *T. remus* and seven *T. rowani* barcodes identified 12 sequences that showed at least 90% sequence identity with at least one of the Treh1-2 or Trwh1-7 haplotypes. Phylogenetic relationships between the BLAST results and the barcodes we obtained were analyzed by neighbor-joining analysis and depicted in a cladogram (Fig. 3). As expected by the high sequence identity, Treh1 and Treh2 clustered with JX559778 and KF365460. The *T. rowani* specimens fell into two groups (Trwh5-7 and Trwh1-4) that were both separable from the *T. remus* haplotypes. These distinctions were consistent with a simple visual inspection of the DNA sequence comparisons (Fig. 2). The *T. rowani* barcode sequences did not cluster with any of the GenBank sequences examined.

Discussion

Fall armyworm populations that overwinter in southern Florida are the main source of infestations in Georgia, South Carolina, and the eastern seaboard states as far north as Pennsylvania (Nagoshi et al. 2012). Therefore, the introduction of new natural enemies capable of reducing the overwintering population prior to migration could provide a cost-efficient and environmentally benign method to mitigate fall armyworm infestations over an extensive geographical region outside the treated area. For this reason, we were interested in characterizing the *Telenomus* species recently found infecting fall armyworm eggs in Florida pasture and cornfields. The collection of *Telenomus* in Levy County in 2009 and Alachua County in 2013 indicates that the species has become established in north-central Florida. This represents the first observation of a permanent *Telenomus* population in North America that has fall armyworm as a preferred host. While the extent of this population in Florida is not known, its presence in the more southern overwintering regions now appears more likely, and justifies more extensive surveys to measure the geographical distribution and population levels in different Florida habitats and times of the year.

DNA barcode comparisons provided strong evidence that the Florida *Telenomus* were related to those found in Ecuador and Honduras previously identified as

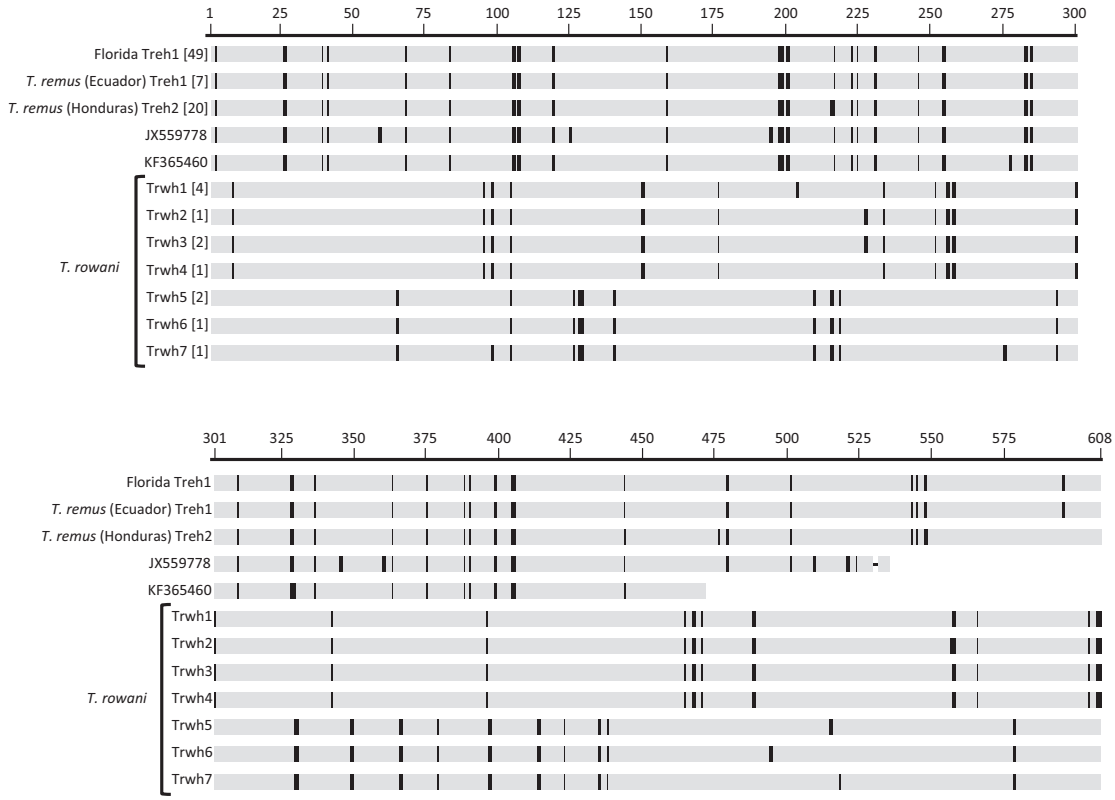

Fig. 2. Diagram of the 608-bp barcode region for the *Telenomus* haplotypes identified in this study, including those from *T. remus* (Treh1-2) and *T. rowani* (Trwh1-7). Comparisons are made with the GenBank sequences JX559778 and KF365460 that had the highest sequence identity (>97%) with both Treh1 and Treh2 as determined by a BLAST search. Numbers in brackets indicate the number of specimens of each type examined. Vertical lines indicate polymorphic sites.

Table 2. Descriptive statistics of polymorphisms found in a 608-bp segment of the COI gene from different *Telenomus* collections

Species (Location)	*n*	Haplotypes	Ps	SS/NS	I/II/III
T. rowani (Ecuador)	12	7	48	47/2	10/1/37
T. remus (Ecuador, Honduras)	27	2	3	3/0	0/0/3
Unknown (Florida)	53	1	0	0/0	0/0/0

n, number of sequences; Ps, number of polymorphic sites; SS, synonymous substitution; NS, nonsynonymous substitution; I/II/III, number of substitutions in the three codon positions.

T. remus. However, the taxonomy of the *Telenomus* species that attack fall armyworm is not conclusive, and until those relationships are clarified and unambiguously identified specimens for each species become available for barcode analysis, discriminating *T. remus* from closely related sibling species will remain problematic. What can be concluded is that the Florida *Telenomus* population is more likely to be *T. remus* than *T. rowani*, and the phylogenetic clustering of the *T. remus*/Florida haplotypes relative to the most similar *Telenomus* and Hymenoptera barcode sequences available in the GenBank database is strongly suggestive that the Honduras, Ecuador, and Florida populations, as well as GenBank sequences JX559778 and KF365460, are of the same species (Fig. 3).

Only two putative *T. remus* haplotypes have been identified, Treh1 in Ecuador and Treh2 in Honduras. This geographical segregation might indicate different origins for these two populations; however, both samplings are from laboratory colonies and so may not be representative of the genetic diversity of the wild populations. However, the field-collected specimens from Florida also showed no genetic variation in the barcode sequence despite multiple collections over four years and from two locations, suggesting that limited genetic variability may be a characteristic of this species in the Western Hemisphere. If the Ecuador and Honduras colonies accurately represent the barcode configurations of their respective local populations, then the observation that all Florida *Telenomus* were Treh1 suggests a closer relationship of Florida populations with *T. remus* from the South American country of Ecuador than those from the Central American country of Honduras. Why this should be the case is unclear and it will be difficult to resolve given the number of *T. remus* mass releases in the region for biological control purposes.

The *T. remus* and Florida collections were compared to *T. rowani* to test the discriminatory power of DNA barcoding relative to a known sibling species. In addition to the morphological keys, the *T. rowani* specimens were almost certainly not *T. remus* given their

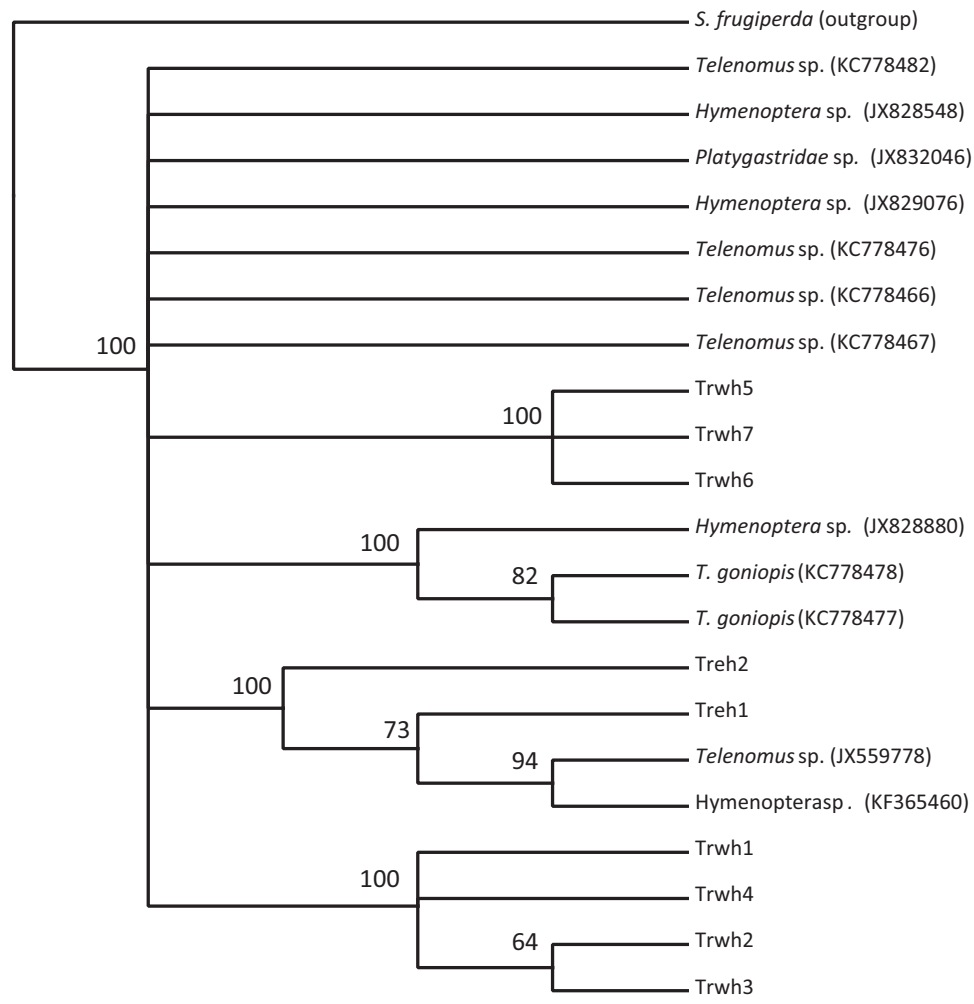

Fig. 3. Cladogram showing phylogenetic relationships between *Telenomus* and related species based on barcode sequence comparisons. In parentheses are the GenBank accession numbers for the 12 sequences that showed greater than 90% sequence identity with at least one of the Treh1-2 and Trwh1-7 haplotypes as determined by BLAST search criteria. Bootstrap resampling with 5,000 replications was performed with numbers indicating bootstrap value and using fall armyworm (*S. frugiperda*) as an outgroup.

development in a host (*Rupella albinella*) not associated with that species. The DNA barcode comparisons were consistent with these assumptions, as all seven *T. rowani* haplotypes clustered separately from the *T. remus*/Florida sequences as seen by cladogram analysis (Fig. 3). In addition, the *T. rowani* haplotypes fell into two distinct clusters, indicating the existence of at least two genetically defined subpopulations. This indicates genetic complexity within the population that has not been distinguished by morphological keys. *T. rowani* is an important egg parasitoid of the rice stem borer *Scirpophaga innotata* (Walker) in Asia (Litsinger et al. 2006), and may have been introduced to the Western Hemisphere for biological control purposes. These results suggest that DNA barcoding may be a particularly useful supplement to morphological keys for the taxonomic categorization of *Telenomus*, which is often problematic due to the small size and physical similarities of the different species. Barcode comparisons can provide a relatively quick approximation of species identity that will increase in precision and accuracy as more DNA sequence information becomes available. Such preliminary results should facilitate subsequent verification by more detailed taxonomic analysis.

In conclusion, these results demonstrate for the first time the establishment of a permanent *Telenomus* population in the United States that targets fall armyworm. The available genetic evidence suggests that the species is *T. remus*, which may be a remnant of mass release studies done in the 1970s, or the result of a subsequent unintended introduction. The identified specimens were collected within 500 km of the fall armyworm overwintering regions in southern Florida, suggesting that *T. remus* might already or soon be established in areas that could mitigate migratory fall armyworm

populations. Facilitating the establishment of a large permanent *Telenomus* population at the overwinter site in southern Florida could prevent the upsurge of fall armyworm migratory populations. However, augmenting the parasitoid density at sites where winter corn is grown would take releases of large numbers of wasps in appropriately timed events (Figueiredo et al. 2002). Before this, research would be needed to understand why the previous releases in the 1970s were not successful and if the sweet corn and other vegetable habitats in southern Florida are appropriate release areas. If successful, releases of *Telenomus* plus the integration of other strategies could provide a cost-effective and environmentally benign method of reducing fall armyworm infestations for a large portion of the eastern United States.

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