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SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Molecular, Morphological, and Biological Differentiation between *Anagrus virlai* sp. n., an Egg Parasitoid of the Corn Leafhopper *Dalbulus maidis* (Hemiptera: Cicadellidae) in the New World, and *Anagrus incarnatus* from the Palaearctic Region (Hymenoptera: Mymaridae)

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Abstract
The common New World egg parasitoid of the corn leafhopper *Dalbulus maidis* (DeLong) (Hemiptera: Cicadellidae), an economically important pest of maize from Argentina to southern USA, has long been misidentified as the Palaearctic species *Anagrus incarnatus* Haliday or its synonym *A. breviphragma* Soyka (Hymenoptera: Mymaridae). Using a combination of genetic and morphometric methods, and available biological information, specimens reared from eggs of *D. maidis* in Argentina and Mexico, described and illustrated here as *Anagrus* (*Anagrus*) *virlai* Triapitsyn sp. n., are shown to be different from those of *A. incarnatus* from the Palaearctic region. Mitochondrial and nuclear ribosomal DNA sequence data provide clear evidence for the separation of the two species. *Anagrus virlai* is also known from Brazil, Colombia, Guadeloupe (France), and Guyana.

Introduction

Anagrus incarnatus Haliday, including its recent synonym *A. breviphragma* Soyka (Hymenoptera: Mymaridae) (Triapitsyn 2015b), has a broad distribution across the Palaearctic region (Triapitsyn 2015a [as *A. breviphragma*]). The true Old World *A. incarnatus*, fairly common in Europe, is an egg parasitoid of the green leafhopper *Cicadella viridis* (L.) (Hemiptera: Cicadellidae), several genera and species of Delphacidae (Hemiptera), and also *Orthotylus virescens* (Douglas & Scott) (Hemiptera: Miridae) (Chiappini 1989, Chiappini *et al* 2015, Triapitsyn & Berezovskiy 2004, Triapitsyn 2015a [as *A. breviphragma*]).

The corn leafhopper *Dalbulus maidis* (DeLong) is an economically important pest of maize from Argentina to the USA, and the Caribbean islands (Nault 1983, 1990, Triplehorn & Nault 1985, Oliveira *et al* 2007, 2013, Virla *et al* 2013). One of its most common egg parasitoids in Argentina and Brazil was tentatively determined by Triapitsyn (1997) as *A. breviphragma* based solely on morphology, albeit with a comment that additional studies, including molecular ones, would be needed to demonstrate correctness of that identification. He has never been comfortable with his identification because specimens from the New World seemingly have a relatively shorter ovipositor and also do not share hosts with the true *A. incarnatus* from the Palaearctic region. They are, of course, also geographically isolated. Furthermore, the common host, *D. maidis*, of the New World *Anagrus Haliday* specimens, has likely co-evolved with its *Zea* spp. plant hosts (Nault & Delong 1980, Nault 1990), which also have a New World origin, so it seemed unlikely that its

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main egg parasitoid would be the same species as that from Europe, i.e., *A. incarnatus*. While fresh, ethanol-preserved specimens from the New World were readily available, getting those from Europe had been difficult until a few specimens of the true *A. incarnartus* were recently collected in Finland and northwestern Russia, and properly preserved for DNA work. Triapitsyn (2015b) clarified the identity of the true *A. incarnatus* based on examination of its lectotype, illustrated here for the first time (Fig 1a, c). Here, we attempt to clarify the mistaken identity of the parasitoids from the Americas using both genetic and morphometric methods. Meanwhile, Hu and Triapitsyn (2016) described *Anagrus* (*Anagrus*) *turpanicus* Triapitsyn & Hu from China. This parasitoid of the leafhopper *Arboridia kakogawana* (Matsumura), a pest of cultivated grapes in the Palaearctic region, is also morphologically similar to the Palaearctic *A. incarnatus*. DNA extracted from one of the paratypes of *A. turpanicus* prior to slide-mounting was successfully sequenced by the second author of the present paper, and it is used here for genetic comparison with both the true *A. incarnatus* from Europe and egg parasitoid of *D. maidis* from Argentina and Mexico. In Argentina, distribution and host associations of "*A. breviphragma*" and later "*A. incarnatus*" were reported by Luft Albarracin *et al* (2005), Virla *et al* (2013), and Luft Albarracin *et al* (2017). In Brazil, "*A. breviphragma*" was recorded parasitizing eggs of *D. maidis* in Sete Lagoas, Minas Gerais (Triapitsyn 1997), Piracicaba, São Paulo (Oliveira and Lopes 2000), and Teresina, Piauí (Querino *et al* 2017). Information on the egg parasitoids of *D. maidis* in Brazil including "*A. breviphragma*" was recently summarized by Querino *et al* (2017). In Mexico, "*A. breviphragma*" (more recently as "*A. incarnatus*") was reported as a main egg parasitoid of *D. maidis* (Moya-Raygoza *et al* 2012, 2014, Moya-Raygoza and Becerra-Chiron 2014) on *Zea* spp., including maize (Moya-Raygoza 2016) and wild teosintes (Moya-Raygoza and Triapitsyn 2017).

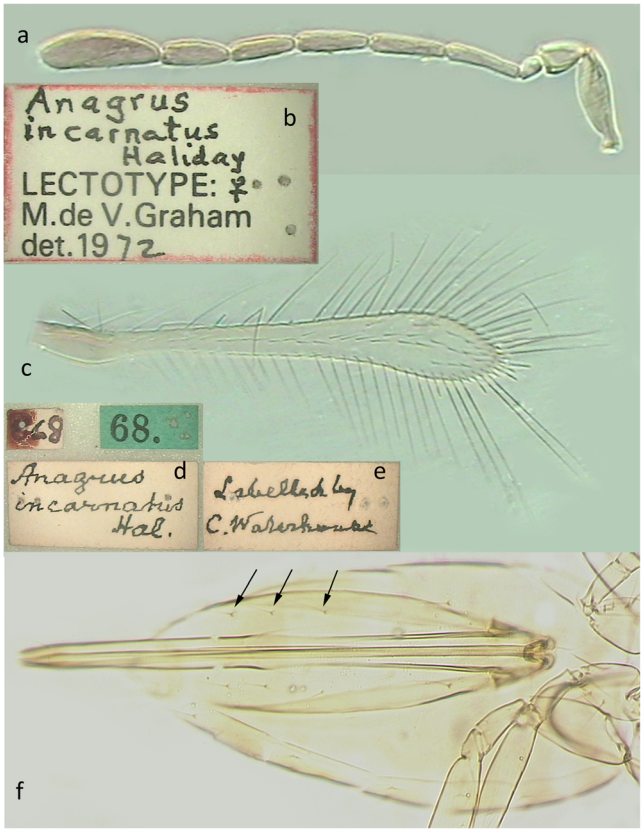

Fig 1 *Anagrus incarnatus* female: **a** antenna (lectotype), **b** lectotype designation label added by M. W. R. de V. Graham, **c** fore wing (lectotype), **d**, **e** numbers added by either F. Enock or C. O. Waterhouse and labels added by C. O. Waterhouse, **f** metasoma (Lazio, Italy); arrows indicate setae on the second valvifer (= external plate of the ovipositor).

Material and Methods

Specimen collection. Essentially the same method was used to rear parasitoids of *D. maidis* in Argentina and Mexico, using sentinel eggs exposed in the cornfields free of pesticide applications during corn growing season (Virla *et al* 2009, Luft Albarracin *et al* 2017). To obtain sentinel eggs, 6–10 females of *D. maidis* were placed in cages (35 cm high × 18 cm diameter) on leaves of potted maize plants in their vegetative stage for oviposition. After two (in Argentina) or three (in Mexico) days, the pots that contained sentinel host eggs were then exposed to parasitism in cornfields for three or four days (in Argentina) or five days (in Mexico). After five (in Mexico) or eight (in Argentina) days, the leaves with exposed eggs were cut from the plant, transported to a rearing room in the laboratory, and transferred to Petri dishes containing wet tissue paper on the bottom and covered with a polyethylene film to avoid desiccation of eggs and leaves, and to contain the emerging parasitoids. Parasitized eggs were checked daily to ensure leaf quality until emergence of all adult parasitoids. Emerging parasitoids were removed from the Petri dishes every day, labeled, and placed in 95% ethanol for storage at − 20°C until they were shipped to the first author. These specimens were used both for the molecular and morphometric analyses and also for taxonomic study (as type material). Specimens of *A. incarnatus* for the molecular analysis were collected either by sweeping (in Finland) or a Malaise trap (in Leningradskaya oblast', Russia). For the morphometric analyses, additional specimens of both species were studied from the collection of the Entomology Research Museum, Department of Entomology, University of California, Riverside, CA, USA.

Taxonomic studies. Morphological analysis is based only on females because males of these two species of *Anagrus* are similar. Other than in genitalic structure, males of *Anagrus* differ from females in the normal sexually dimorphic

features: flagellum filiform, body color usually at least a little darker, and fore wing usually a little wider. Identification of males to species is often difficult or even impossible morphologically without rearing them together or associating them through collecting the sexes together at one time and place. Therefore, often it is not the species that is differentiated but only the females of that species.

Results of the genetic analysis were key in guiding the separation of the new species of *Anagrus* from the Old World *A. incarnatus* based on morphometry of females.

For the descriptions, the morphological terms of Gibson (1997) were used. All measurements (as length or length: width for the wings) are given in micrometers (μm). Abbreviations used in the key and description are as follows: F = antennal funicular (female) or flagellar (male) segment; mps = multiporous plate sensillum or sensilla on the antennal flagellar segments (= longitudinal sensillum or sensilla or sensory ridge(s) of other authors).

Specimens from ethanol were dried using a critical point drier, then point-mounted and labeled. Selected specimens were further dissected and slide-mounted in Canada balsam. Slide mounts were examined under a Zeiss Axioskop 2 plus compound microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) and photographed using the Auto-Montage® system (Syncroscopy, Princeton, NJ, USA). Photographs were retouched where necessary using Adobe Photoshop® (Adobe Systems, Inc., San Jose, CA, USA).

Specimens examined are deposited in the following collections: CNC, Canadian National Collection of Insects, Arachnids and Nematodes, Ottawa, Ontario, Canada; CZUG, insect collection of Centro de Estudios en Zoología, Universidad de Guadalajara, Las Agujas, Zapopan, Jalisco, Mexico; EMNB, Embrapa Meio-Norte (Mid-North), Empresa Brasileira de Pesquisa Agropecuária, Teresina, Piauí, Brazil; IMLA, Fundación e Instituto Miguel Lillo, San Miguel de Tucumán, Tucumán, Argentina; MLPA, Museo de La Plata, La Plata, Buenos Aires, Argentina; NMID, the National Museum of Ireland – Natural History, Dublin, Ireland; and UCRC, Entomology Research Museum, Department of Entomology, University of California, Riverside, CA, USA.

DNA extraction, amplification, and sequencing. DNA was extracted from individual wasps using the "HotSHOT" method of Truett *et al* (2000), in a total volume of 80 μL. This non-destructive method allowed for the recovery and slide-mounting of each specimen following extraction; each slide was then labeled with the assigned P. F. Rugman-Jones' primary molecular voucher PR number and UCRC database UCRC ENT number. Our sample included "*incarnatus*-like" wasps form both the Old World (Finland and Russia) and New World (Mexico and Argentina). DNA was also extracted from three further taxa in the *incarnatus* species group of *Anagrus*: *A. avalae* Soyka (UK, England, Kent Co., Sevenoaks, viii.2014, A. Polaszek [1 female, molecular voucher PR15-026, UCRC ENT 311795]); *A. fennicus* Soyka (RUSSIA, Moskovskaya oblast', Balashikhinskiy rayon, Chyornoe, 2.v.2016, A. K. Akhatov, emerged from old leaves of *Carex* sp. [1 female, molecular voucher PR16-512, UCRC ENT 311796]); and *A. turpanicus* (CHINA, Xinjiang, Turpan, Ya'er Guolecun, 42°56′43″N 89°05″37′E, 53 m, 9.vii.2014, W.-q. Cao (Cao Wen-qiu), from eggs of *Arboridia kakogawana* (Matsumura) on table grape leaves [1 female paratype, molecular voucher PR15-021, UCRC ENT 249980]). *Anagrus avalae* and *A. fennicus* both have a pair of adnotaular setae on the midlobe of the mesoscutum, which are lacking in the members of the *A. incarnatus* species complex such as *A. incarnatus*, *A. turpanicus*, and the new species of *Anagrus* from the New World described herein.

The polymerase chain reaction (PCR) was employed to amplify the "barcoding" region of the mitochondrial cytochrome c oxidase subunit I gene (COI) using LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′; Folmer *et al* 1994), as described in Rugman-Jones *et al* (2012). In a separate PCR, the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal RNA (rRNA) was also amplified using the primers, 58SF (5′-GTGAACTGCAGGACACATGAAC-3′) (Porter and Collins 1991) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al* 1990), as described in Morse *et al* (2016). All reactions were performed in 25-μL volumes on a Mastercycler® ep gradient S thermocycler (Eppendorf North America Inc., New York, NY, USA). Amplification was confirmed by gel electrophoresis and PCR products were cleaned using a DNA Clean & Concentrator™-5 kit (Zymo Research Corporation, Irvine, CA, USA). Amplicons were direct sequenced in both directions at the Institute for Integrative Genome Biology, University of California at Riverside. Coincidentally, direct sequencing failed for the ITS2 amplicon of *A. avalae* (PR15-026), *A. fennicus* (PR16-512), and *A. turpanicus* (PR15-021). ITS2 is part of the ribosomal cistron, of which there are hundreds of tandem copies in a typical eukaryote genome. Although rare, this creates potential for sequence variation among copies (Buckler *et al* 1997). To overcome this problem, sequencing of these three taxa was facilitated by cloning. The ITS2 PCR product was inserted into a plasmid vector (pGEM-T Easy Vector System; Promega) and plasmids were transformed in JM109 competent cells. For each taxon, three insert-positive clones were subsequently amplified and sequenced using M13 primers. A consensus of the three clones was then taken for each of these three species.

The parity of forward and reverse reads was checked using SEQUENCHER 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA) and priming regions were removed manually in BioEdit version 7.0.5.3 (Hall 1999). The online tool EMBOSS Transeq (Rice *et al* 2000) was used to translate all protein

coding COI sequences into their amino acid chains, confirming the absence of indels and pseudogenes in the final dataset. All sequences were deposited in GenBank® (Benson *et al* 2008).

Genetic analysis. COI and ITS2 sequences were aligned separately using MAFFT version 7.050 (Katoh & Standley 2013) and the Q-INS-i algorithm with default settings. Phylogenetic inference from ITS2 is typically problematic due to large interspecific differences that make alignment of this region difficult and somewhat ambiguous. Therefore, relationships among our specimens were empirically assessed based only on variation in COI. In turn, ITS2 sequences were examined "by eye" to determine if corroborating patterns were present.

The aligned COI dataset contained 18 terminal taxa (including outgroups), 587 nucleotide positions, and no gaps. Genetic variation among our sequences was estimated by calculating uncorrected *p*-distances between all possible sequence pairs, using MEGA version 6 (Tamura *et al* 2013). All ambiguous positions were removed for each sequence pair. MEGA was also used to investigate relationships among the sequences by constructing a neighbor-joining (NJ) tree based on the *p*-distances. Branch support was estimated using a bootstrap procedure with 1000 replicates.

Results

Taxonomy

Anagrus (Anagrus) incarnatus Haliday 1833

Anagrus incarnatus Haliday 1833: 347. Type locality: not specified in the original description (certainly in Europe, most likely in UK or Ireland). Lectotype female (Fig 1a–e), designated by Graham 1982: 200 (NMID), examined (Triapitsyn 2015b). Illustrated here are some of its non-original labels (Fig 1b, d, e), detailed information on which was given in Triapitsyn (2015b).

Anagrus breviphragma Soyka 1956: 25. Type locality unknown (certainly in Europe): (Chiappini 1989). Lectotype female (NHMW), de-facto designated by Chiappini 1989: 105–106 (mentioned as holotype), examined by the senior author during several visits to the NHMW. Synonymized under *A. incarnatus* by Triapitsyn 2015b: 216.

Anagrus breviphragma Soyka: Chiappini 1989: 105–106 (synonymy, type information, diagnosis), Chiappini *et al* 1996: 558–559 (key, illustration), Triapitsyn 1997: 7 (in part: distribution), Chiappini *et al* 1999: 393–398 (genetic identity), Chiappini and Mazzoni 2000: 1663, 1670, 1672 (morphology of male genitalia), Triapitsyn 2000: 214–215 (in part: distribution, host associations), Chiappini 2002: 261–264 (development, illustrations); Chiappini & Solinas 2002: 267–271 (morphology), Triapitsyn & Berezovskiy 2004: 15–16 (distribution, host associations), Triapitsyn 2015a: 12 (key), 19, 25 (illustrations), 27–28 (coded redescription of Palaearctic specimens only, distribution [in part], hosts [in part]), 33 (illustration), 41 (discussion), 42 (list).

Anagrus incarnatus Haliday: Chiappini 1989: 112–113 (lectotype only), Triapitsyn 2015b: 216 (taxonomic history, list of synonyms, type information, discussion).

Material examined. Measured specimens (all in UCRC). CHINA, Beijing, Fragrant Hills (Xiangshan) Park, 23–24.vii.2002, M. L. Buffington [1 female]. ITALY: Emilia-Romagna, Piecenza Province, Piacenza, E. Chiappini: 20.ii.1984, emerged from an egg in *Carex* sp. [1 female]; Mortizza, 30.x.1985, emerged from egg of *Cicadella viridis* [2 females]. Lazio, Roma Province, Castelporziano Presidential Estate, Fosso di Trafusina, 41°46.670′N 12°24.751′E, 30 m, 11–12.vi.2003, M. Bologna, J. Munro, A. Owen, J. D. Pinto [1 female]. RUSSIA, Primorskiy kray, Ussuriyskiy rayon, Gornotayozhnoye, M. V. Michailovskaya: 8.vi.1999 [1 female]; 25–26.ix.1999 [1 female]; 1–10.viii.2000 [1 female]; 5–8.x.2000 [3 females]; 9–12.x.2000 [4 females].

Molecular vouchers (all in UCRC). FINLAND, Tavastia Proper, Janakkala, Kalpalinna, 60°55′37″N 24°35′49″E, 101 m, 8.vii.2017, S. V. Triapitsyn, V. Vikberg [1 female, PR17-594, UCRC ENT 251157]. RUSSIA, Leningradskaya oblast', Vsevolozhskiy rayon, Vaganovo, 60°05′24.5″N 31°02′08.3″E, 25 m, 15–30.vi.2016, A. Knyshov [2 females, PR16-371 (UCRC ENT 495130) and PR16-374 (UCRC ENT 249949)].

Brief diagnosis (updated from Triapitsyn 2015a). Member of the *incarnatus* species complex of the *incarnatus* species group of *Anagrus* (*Anagrus*). Female antenna (Fig 1a) with F2 the longest funicular segment; mps on F3 (1), F4 (1), F5 (2), F6 (2), and clava (5); midlobe of mesoscutum without adnotaular setae; apex of fore wing disc with a distinct, large bare area at posterior margin subapically (Fig 1c); ovipositor (Fig 1f) 2.6–3.3× as long as protibia length; external plate of ovipositor with 3 setae.

Distribution. Palaearctic region from the UK to Japan. In addition to the 15 countries listed in Triapitsyn (1997, 2002), Triapitsyn & Berezovskiy (2004), and Triapitsyn (2015a), we add Finland (new record, because all the previous records of this species from there were of *A. incarnatosimilis* Soyka).

Hosts. Cicadella viridis (Cicadellidae), *Conomelus anceps* (Germar), *Dicranotropis hamata* (Boheman), *Muellerianella fairmairei* (Perris) (Delphacidae), and *Orthotylus virescens* (Douglas & Scott) (Miridae) (Moratorio & Chiappini 1995, Triapitsyn & Berezovskiy 2004, Chiappini *et al* 2015,

Triapitsyn 2015a [as *A. breviphragma*, from the Palaearctic region only]).

Biology. Biological traits and other aspects of *A. incarnatus* [as *A. breviphragma*] in Europe were studied by Moratorio and Chiappini (1995), Chiappini (2002), and Chiappini *et al* (2012, 2015). This species is a facultative gregarious parasitoid, with 5–8 adults emerging from larger leafhopper eggs, whereas only one adult develops in smaller planthopper (Delphacidae) eggs (Moratorio and Chiappini 1995, Chiappini *et al* 2015).

Anagrus (Anagrus) virlai Triapitsyn, sp. n.

Anagrus breviphragma Soyka (misidentifications): Triapitsyn 1997: 2 (key), 6–7 (illustrations of the specimens from Argentina, distribution [in part], host associations [in part], discussion); Oliveira and Lopes 2000: 265–267 (distribution and egg parasitism of *D. maidis* in Brazil), Triapitsyn 2000: 214–215 (key, distribution [in part], host associations [in part]), Virla 2001: 240–246 (host associations, biology), Triapitsyn 2002: 215 (key), 217 (distribution, host association), Luft Albarracin *et al* 2005: 258–259 (host association in Argentina), Luft Albarracin *et al* 2009: 5 (distribution and host associations in Argentina), Virla *et al* 2009: 508–509 (host associations, distribution in Mexico), Moya-Raygoza *et al* 2012: 107–110 (egg parasitism of *D. maidis* in Argentina and Mexico), Virla *et al* 2013: 4–5 (egg parasitism of *D. maidis* and distribution in Argentina), Moya-Raygoza *et al* 2014: 310 (host associations), Moya-Raygoza and Becerra-Chiron 2014: 927 (mentioned as egg parasitoid of *D. maidis* in Mexico), Moya-Raygoza and Triapitsyn 2015: 296–297 (host associations), Querino *et al* 2017: 124–126 (historical and biological information), 128 (brief diagnosis, distribution in Brazil, host associations), 129 (illustrations of female from Brazil) [pages differ in the Portuguese version].

Anagrus incarnatus Haliday (misidentifications): Moya-Raygoza 2016: 725–728 (host association in Mexico), Moya-Raygoza and Triapitsyn 2017: 694–696 (host association, distribution in Mexico, taxonomic notes on female, illustrations of female), Luft Albarracin *et al* 2017: 670–674 (host associations, key, distribution in Argentina, illustrations).

Type material. Holotype female, deposited in UCRC, on slide (Fig 2a) labeled: 1. "MEXICO: Jalisco Zapopan 20.viii.2009 G. Moya-Raygoza vial #5"; 2. "Mounted by V. V. Berezovskiy 2014 in Canada balsam"; 3. "From eggs of *Dalbulus maidis* (DeLong and Wolcott) on maize, *Zea mays* spp. *mays*"; 4. "*Anagrus breviphragma* Soyka Det. S. TRIAPITSYN 2014"; 5. [magenta] "*Anagrus* (*Anagrus*) *virlai* Triapitsyn HOLOTYPE ♀"; 7. [barcode database label] "UCRC UCRC ENT 311846". The holotype is in good condition, dissected under 3

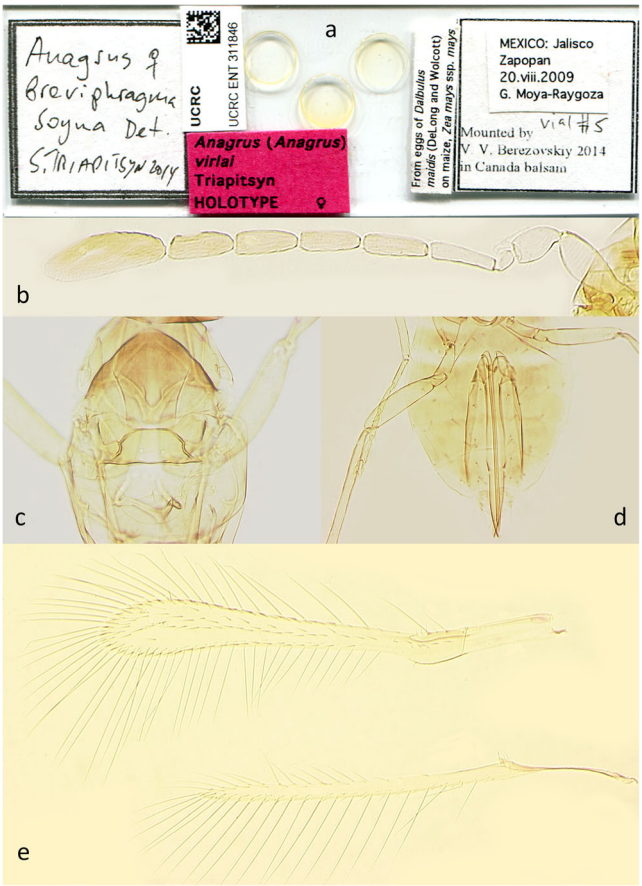

Fig 2 *Anagrus virlai* sp. n. female: **a** slide (holotype), **b** antenna (holotype), **c** mesoscutum (paratype from Las Talitas, Tucumán, Argentina), **d** metasoma (paratype from San Miguel de Tucumán, Tucumán, Argentina), **e** fore and hind wings (holotype).

coverslips. Along with some paratypes, the holotype was measured for the morphometric analysis.

Paratypes. Measured specimens (all on slides in UCRC). ARGENTINA: Córdoba: Barranca Yaco, 2–6.iv.2015, E. Luft Albarracin (from eggs of *D. maidis* on maize) [1 female, molecular voucher PR16-370, UCRC ENT 143822]. Río Cuarto, 7–15.xi.1994, E. G. Virla (from eggs of *Delphacodes kuscheli* Fennah on oat) [1 female]. Mendoza, San Rafael, xi.2005, E. Luft Albarracin (from eggs of Agallinae leafhoppers) [2 females]. Tucumán: El Manantial, 26°49′50.2″S 65°16′59.4″ W, 495 m, i.2005, E. Luft Albarracin (from eggs of *D. maidis* on maize) [1 female]. Estación Aráoz, 21–28.v.2015, E. Luft Albarracin (from eggs of *D. maidis* on maize) [1 female, molecular voucher PR16-366, UCRC ENT 495104]. Las Talitas, iv.2005, E. Luft Albarracin (from eggs of *D. maidis* on maize) [1 female]. San Miguel de Tucumán, iii.2005, E. Luft Albarracin (from eggs of *Chlorotettix fraterculus* (Berg)) [1 female]. BRAZIL, Minas Gerais, Sete Lagoas, 5.vi.1996, D. L. Q. Santana (from eggs of *D. maidis* on maize) [2 females]. MEXICO, Jalisco, Zapopan, G. Moya-Raygoza (from eggs of *D. maidis* on maize): 10.ix.2008 [1 female]; 22.viii.2015 [1 female, molecular voucher PR15-507, UCRC ENT 249672];

25.viii.2015 [1 female, molecular voucher PR15-502, UCRC ENT 249950]; 23.ix.2015 [1 female, molecular voucher PR15-503, UCRC ENT 249952]; 25.ix.2015 [1 female, molecular voucher PR15-506, UCRC ENT 249939].

Other paratypes (all on points). ARGENTINA, Tucumán: Ranchillos y San Miguel, 21–28.v.2015, E. Luft Albarracin (from eggs of *D. maidis*) [1 female, IMLA; 1 female, MLPA, 1 female, UCRC]. San Miguel de Tucumán, 8–12.iii.2015, E. Luft Albarracin (from eggs of *D. maidis*) [1 female, IMLA]. Tafí del Valle, i.2014, E. Luft Albarracin (from eggs of *D. maidis*) [1 female, IMLA; 1 female, MLPA]. MEXICO, Jalisco, Zapopan, G. Moya-Raygoza (from eggs of *D. maidis* on maize): 10.ix.2008 [10 females, 5 males: 2 females, 1 male, CNC; 2 females, 2 males, CZUG; 6 females, 2 males, UCRC].

Other paratypes (all on slides in UCRC). ARGENTINA: Córdoba, Río Cuarto, 7–15.xi.1994, E. G. Virla (from eggs of *Delphacodes kuscheli* Fennah on oat) [4 females, 1 male]. Salta, Cabeza de Buey, 17.v.1994, E. G. Virla (from eggs of *D. maidis* on maize) [3 females]. Tucumán: El Manantial, 26°49′50.2″S 65°16′59.4″W, 495 m, i.2005, E. Luft Albarracin (from eggs of *D. maidis* on maize) [2 females]. San Miguel de Tucumán, iii.2004, E. Luft Albarracin, (from eggs of *Chlorotettix* sp. on maize) [1 female]. BRAZIL, Minas Gerais, Sete Lagoas, 5.vi.1996, D. L. Q. Santana (from eggs of *D. maidis* on maize) [4 females]. MEXICO, Jalisco: Ejutla, 2.ix.2015, G. Moya-Raygoza (from eggs of *D. maidis* on *Zea mays* ssp. *parviglumis*) [1 female]. Piedra Ancha, 10.x.2014, G. Moya-Raygoza (from eggs of *D. maidis* on perennial teosinte, *Zea perennis*) [1 female]. Zapopan, G. Moya-Raygoza (from eggs of *D. maidis* on maize): 10.ix.2008 [1 male]; 20.viii.2009 [1 male].

Other paratypes, molecular vouchers (all on slides in UCRC). ARGENTINA: Córdoba, Barranca Yaco, 2–6.iv.2015, E. Luft Albarracin (from eggs of *D. maidis* on maize) [1 female, PR16-369, UCRC ENT 249978]. Tucumán, Estación Aráoz, 21–28.v.2015, E. Luft Albarracin (from eggs of *D. maidis* on maize) [4 females, PR15-291 (UCRC ENT 249932), PR15-292 (UCRC ENT 301942), PR16-367 (UCRC ENT 302271), PR16-368 (UCRC ENT 311831)]. MEXICO, Jalisco, Zapopan, G. Moya-Raygoza (from eggs of *D. maidis* on maize): 10.ix.2008 [3 females, PR15-027, PR15-037 and PR15-039 (extracts failed)]; 22.viii.2015 [1 male, PR15-505, UCRC ENT 311829].

Other, non-type, material examined. ARGENTINA: Córdoba: Barranca Yaco, 1–4.xii.2015, E. Luft Albarracin (from eggs of *P. maidis* on maize) [3 females, 1 male, IMLA]. Espinillos, 12–15.xii.2016, E. Luft Albarracin (from eggs of *D. maidis* on maize) [2 females, 1 male, IMLA]. Río Cuarto, 12–15.xii.2016, E. Luft Albarracin (from eggs of *D. maidis* on maize) [3 females, 2 males, IMLA]. Tucumán: El Manantial, 24.xi.2011, E. Luft Albarracin (from eggs of *D. maidis* on maize) [2 females, IMLA]. La Virginia, 8–16.xii.2009, E. Luft Albarracin (from eggs of *D. maidis* on maize) [1 female, IMLA; 1 female, MLPA]. Los Nogales, E. Luft Albarracin (from eggs of *D. maidis* on maize): 21–28.xii.2006 [4 females, 2 males, IMLA]; 8–12.ii.2007 [3 females, 2 males, IMLA]. San Miguel de Tucumán: [no date, likely in mid 1990s], E. G. Virla (from a laboratory colony at CIRPON on eggs of *D. maidis*, originally from Salta, Cabeza de Buey, 17.v.1994, E. G. Virla, from sentinel eggs of *D. maidis* on maize (Virla 2001)) [1 female, UCRC]; viii.2004, E. Luft Albarracin (from eggs of *D. maidis* on maize) [2 females, IMLA]; iii.2005, E. Luft Albarracin (from eggs of *Chlorotettix fraterculus* (Berg)) [1 female, UCRC (specimen lacks antennae)]; 20–26.xi.2010, E. Luft Albarracin [1 female, IMLA]; 13–23.x.2011, E. Luft Albarracin [1 female, IMLA]; 2–7.xii.2015, E. Luft Albarracin [2 females, 1 male, IMLA]; 17–22.ii.2016, E. Luft Albarracin [2 females, 2 males, IMLA]; 23–28.ii.2011, E. Luft Albarracin (from eggs of *Peregrinus maidis* (Ashmead) on maize) [1 female, 1 male, IMLA]; 4–12.xii.2014, E. Luft Albarracin [2 females, 1 male, IMLA]; 23–28.xi.2015, E. Luft Albarracin [3 females, 2 males, IMLA]. BRAZIL: Minas Gerais, Sete Lagoas, 5.vi.1996, D. L. Q. Santana (from eggs of *D. maidis* on maize) [1 female, UCRC (specimen is damaged)]. Piauí, Teresina, ii-iv.2013, A. R. Meneses (from sentinel eggs of *D. maidis* on maize) [15 females, EMNB]. MEXICO, Jalisco, Zapopan, 22.viii.2015, G. Moya-Raygoza (from eggs of *D. maidis* on maize) [10 females, CZUG]. These additional specimens are not included in the type series because many of them are either preserved in ethanol or a few are not mounted well enough; most of them have not been examined by the senior author.

Diagnosis. Member of the *incarnatus* species group of the nominate subgenus of *Anagrus*, as defined by Chiappini *et al* (1996). Female antenna (Fig 2b) with F2 the longest funicular segment; mps on F3 (1), F4 (1), F5 (1), F6 (2), and clava (5); midlobe of mesoscutum without adnotaular setae (Fig 2c); fore wing (Fig 2e) disc with a distinct, large subapical bare area; ovipositor 1.8–2.3× length of protibia.

Morphologically, *A. virlai* is most similar to the Palaearctic *A. incarnatus*, to which [as *A. breviphragma*] it keys in Triapitsyn (2015a) but the latter has a relatively longer ovipositor and 2 mps on F5 of the female antenna. The morphometric analysis (Table 1) clearly separates *A. virlai* from *A. incarnatus* based on the ratio of ovipositor length to protibia length: at most 2.3× in *A. virlai* compared to at least 2.6× in *A. incarnatus*. The ovipositor length is also usually notably different, with just a slight overlap (Table 1) due to one small specimen of *A. incarnatus* from Beijing, China: that in *A. virlai* is shorter on average (0.269 ± 0.14 mm) versus 0.353 ± 0.03 in *A. incarnatus*. All other measurements between both sexes of these two taxa overlap significantly and the observed morphological features are quite similar.

The new species is also similar to *A. turpanicus* from Xinjiang, China, in which, however, F2 of the female antenna is at most as long as F3 and always shorter than the following

Table 1 Measurements (in micrometers, µm) and ratios of some diagnostically important morphological features in slide-mounted females of *Anagrus incarnatus* from the Old World and *Anagrus virlai* from the New World (mean and standard deviation (SD) are given in parentheses, *n* = 14 for both species).

Species	Body length range (mean/SD)	Ratio of fore wing length: width (mean/SD)	Ovipositor length range (mean/SD)	Ratio of ovipositor length: protibia length (mean/SD)	F2 length range (mean/SD)
Anagrus incarnatus	529–891 (676/82)	7.2–10.5 (8.7/0.9)	290–412 (353/30)	2.6–3.3 (2.9/0.2)	50–86 (70/10)
Anagrus virlai	504–609 (555/36)	7.7–9.4 (8.2/0.5)	246–295 (269/14)	1.8–2.3 (2.1/0.1)	58–73 (63/4)

funicular segments, whereas F5 bears just 1 mps and the ovipositor is at most 2.4× (2.1–2.4×) length of protibia (Hu & Triapitsyn 2016). All three species also differ genetically.

Description. Female (holotype and paratypes). Body length of dry-mounted, critical point-dried paratypes 400–600 µm, and of the slide-mounted paratypes 500–700 µm. Body usually mostly light brown except anterior half or so of mesoscutum brown and frenum of scutellum and propodeum whitish, but often gaster either partially (basally only) or entirely brown; scape, pedicel and F1 yellowish or very light brown, rest of flagellum brown; legs mostly yellowish-brown, wings hyaline. Antenna (Fig 2b) with scape 2.8–3.3× as long as wide, with cross-ridges, 1.9–2.1× length of pedicel; F1 slightly longer than wide, a little less than half of pedicel length; F2 the longest funicular segment (slightly longer than more or less subequal following funicular segments); mps on F3 (1), F4 (1); F5 (1), and F6 (2); clava with 5 mps, 2.8–3.6× as long as wide, usually slightly shorter than but rarely almost as long as combined length of F5 and F6. Midlobe of mesoscutum without adnotaular setae (Fig 2c). Fore wing (Fig 2e) 7.5–9.4× as long as wide, longest marginal seta 2.3–2.6× maximum wing width; distal macrochaeta 1.6–1.9× length of proximal macrochaeta; disc with several rows of setae in addition to admarginal rows of setae (1 complete row originating behind apex of venation and about 1 to 2 irregular rows in the broadest part of disc), leaving a distinct subapical bare area at posterior margin. Hind wing (Fig 2e) 26–27× as long as wide, longest marginal seta 6.6–7.0× maximum wing width; disc mostly bare except for incomplete admarginal rows of setae. Ovipositor (Fig 2d) not extending anteriorly to (and thus not overlapping) mesophragma in slide-mounted specimens, and only exserted a little beyond apex of gaster posteriorly (by 0.06–0.13× total ovipositor length). Second valvifers (= external plates of ovipositor), e.g., Chiappini (1989) and Chiappini *et al* (1996), each with 3 setae (Fig 2d). Ovipositor 1.8–2.3× length of protibia (2.2× in the holotype).

Measurements (µm) of the holotype (as length or length: width). Mesosoma 236; gaster 282; ovipositor 257. Antenna: scape 79; pedicel 42; F1 17; F2 58; F3 55; F4 53; F5 54; F6 55; clava 106. Fore wing 551:67; longest marginal seta 173. Hind wing 520:20; longest marginal seta 139.

Male (paratypes). Body length of the dry-mounted, critical point-dried paratypes 490–630 µm, and of the slide-mounted paratypes 615–730 mm. Body mostly brown except frenum of scutellum and propodeum whitish, appendages light brown. Scape 2.7–3.2× as long as wide, F1 shorter and a little wider than following flagellomeres (Fig 3a). Fore wing (Fig 3b) 6.7–7.2× as long as wide. Genitalia (Fig 3c) length 133–142 µm.

Etymology. This new species is named in honor of our colleague and friend Eduardo G. Virla (Investigador de CONICET, Instituto de Entomología, Fundación Miguel Lillo, San Miguel de Tucumán, Argentina) who first reared it from eggs of *D. maidis* and also studied its biology (Virla 2001).

Distribution. Nearctic region: Mexico (Virla *et al* 2009, Moya-Raygoza *et al* 2012, 2014, Triapitsyn 2015a [as *A. breviphragma*]), Neotropical region: Argentina, Brazil, Colombia, Guadeloupe (France), and Guyana (Triapitsyn 1997, 2002, 2015a [as *A. breviphragma*]).

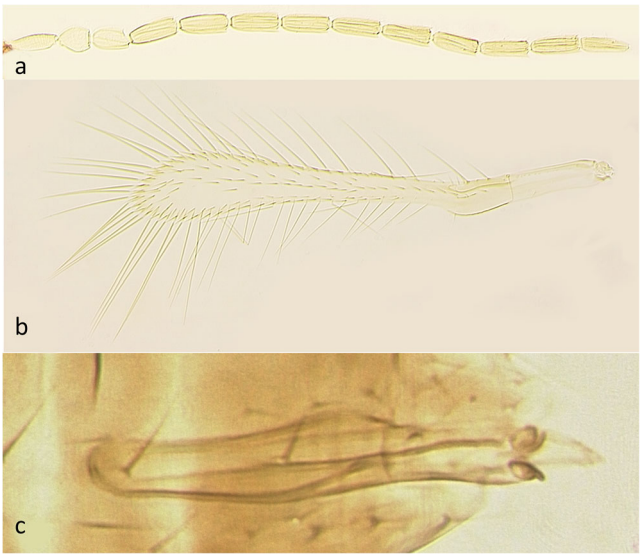

Fig 3 *Anagrus virlai* sp. n. male (paratypes from Zapopan, Jalisco, Mexico): **a** antenna, **b** fore wing, **c** genitalia.

Table 2 Genetic divergence between (below diagonal) five species of *Anagrus*, based on a 587-bp fragment of the mitochondrial COI gene. Diagonal element shows intraspecific variation (*Anagrus incarnatus* and *Anagrus virlai* only). Average pairwise uncorrected *p*-distances calculated using MEGA6.

	incarnatus	*virlai*	*avalae*	*fennicus*	*turpanicus*
incarnatus	0.002				
virlai	0.052	0.001			
avalae	0.060	0.060	–		
fennicus	0.067	0.059	0.058	–	
turpanicus	0.060	0.052	0.063	0.079	–

Hosts. *Agalliana ensigera* Oman, *Chlorotettix fraterculus* (Berg), *Ciminius platensis* (Berg), *Dalbulus elimatus* (Ball), *Dalbulus maidis* (DeLong), *Dechacona missionum* (Berg), *Exitianus obscurinervis* (Stål), *Hortensia similis* (Walker), *Xerophloea viridis* (Fabricius) (Cicadellidae), as well as *Delphacodes kuscheli* Fennah and *Peregrinus maidis* (Ashmead) (Delphacidae) (Triapitsyn 1997, Virla 2001; Luft Albarracin *et al* 2009, Virla *et al* 2009, Moya-Raygoza *et al* 2012, 2014, Triapitsyn 2015a, Querino *et al* 2017 [as *A. breviphragma*], Luft Albarracin *et al* 2017 [as *A. incarnatus*]).

Biology. In eggs of *D. maidis*, *A. virlai* always develops as a solitary parasitoid (Virla 2001), as it does in those of its other leafhopper and planthopper hosts known in Argentina (E. Luft Albarracin, unpublished). Virla (2001) provided a detailed account of its other biological traits using eggs of *D. maidis* as host.

Results of the molecular analyses

Sequences of the COI gene provided strong evidence that *A. virlai* is distinct from *A. incarnatus*. Two haplotypes were identified for each species, with those from Old World, i.e., *A. incarnatus*, being 5.2% divergent from those from the New World, i.e., *A. virlai*, based on uncorrected *p*-distances (Table 2, Fig 4). This compared favorably with levels of divergence between other accepted species in the *incarnatus* species group (Table 2, Fig 4). In contrast, variation within each of the two species (*A. incarnatus* and *A. virlai*) was very low (Table 2). Specimens of *A. virlai* from Mexico ($n = 5$) all shared a single haplotype which differed at only a single nucleotide position from the second haplotype, shared by all specimens from Argentina ($n = 7$; Fig 4). However, this substitution (T to G at position 58) resulted in a non-synonymous change in the coded amino acid chain (from serine to alanine). Similarly, the COI nucleotide sequence of the *A. incarnatus* specimen from Finland (PR17-594) differed from that shared by the two specimens from Russia at only two positions (342 and 384). In this case, both are considered to be synonymous changes.

The patterns found in the mitochondrial COI sequences were also repeated in the nuclear ribosomal ITS2 locus. The ITS2 amplicon from all 12 *A. virlai* specimens was 638 bp long,

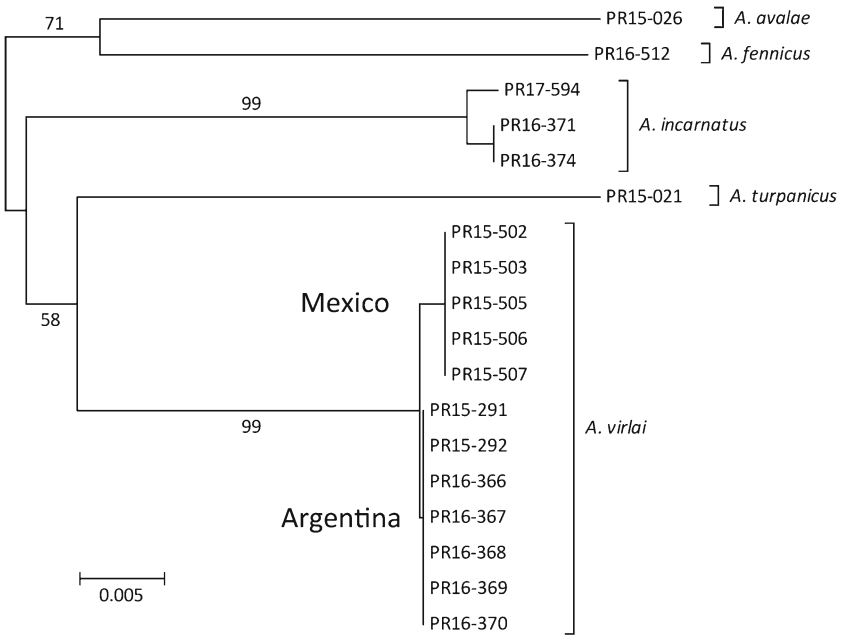

Fig 4 Relationships among the sequences of a 587-bp fragment of COI from five species of *Anagrus*. Optimal unrooted NJ tree with the sum of branch length = 0.11945095. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches and the tree is drawn to scale, with branch lengths indicating uncorrected *p*-distance.

whereas that from three *A. incarnatus* specimens showed a small amount of length variation (613–621 bp), as a result of differing numbers of repeats at several poly-A or poly-T sites (Table 3). The MAFFT alignment of *A. incarnatus* and *A. virlai* sequences resulted in a matrix with 658 nucleotide positions, typified by large numbers of substitutions and indels between the two species (Table 3). Within *A. virlai*, the ITS2 sequence differed very slightly between specimens from Mexico and

Table 3 MAFFT alignment of ITS2 sequences highlighting the differences between *Anagrus incarnatus* (*A. inc.*) and *Anagrus virlai*. The aligned matrix contains 658 positions and variable positions (indels and substitutions) are in bold and designated by asterisks. Intraspecific variation between *Anagrus virlai* from Argentina and Mexico is further highlighted with shading.

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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                             10        20        30        40        50        60        70
A. inc. PR17-594    ATCGACATTT CGAACGCACA TTGCGGTCCA CGGATTTAAT TCCCGGACCA CGCCTGGCTG AGGGTCGTTT
A. inc. PR16-371    ATCGACATTT CGAACGCACA TTGCGGTCCA CGGATTTAAT TCCCGGACCA CGCCTGGCTG AGGGTCGTTT
A. inc. PR16-374    ATCGACATTT CGAACGCACA TTGCGGTCCA CGGATTTAAT TCCCGGACCA CGCCTGGCTG AGGGTCGTTT
A. virlai [Arg]     ATCGACATTT CGAACGCACA TTGCGGTCCA CGGATTTAAT TCCCGGACCA CGCCTGGCTG AGGGTCGTTT
A. virlai [Mex]     ATCGACATTT CGAACGCACA TTGCGGTCCA CGGATTTAAT TCCCGGACCA CGCCTGGCTG AGGGTCGTTT

                    ....|....| ....|....| ...*|...*| ..*.|****| ....*....* *...|....| ....|....|
                             80        90        100       110       120       130       140
A. inc. PR17-594    ATAAATATAA ACCAGACTGC TTGMGGGTGT TGCGT----T TTATRCGCAA CGCACCAAGC GATACCTGAA
A. inc. PR16-371    ATAAATATAA ACCAGACTGC TTGAGGGTGT TGCGT----T TTATACGCAA CGCACCAAGC GATACCTGAA
A. inc. PR16-374    ATAAATATAA ACCAGACTGC TTGAGGGTGT TGCGT----T TTATACGCAA CGCACCAAGC GATACCTGAA
A. virlai [Arg]     ATAAATATAA ACCAGACTGC TTGCGGGTAT TGTGTATTTT TTATACGCAT TGCACCAAGC GATACCTGAA
A. virlai [Mex]     ATAAATATAA ACCAGACTGC TTGCGGGTAT TGTGTATTTT TTATACGCAA CGCACCAAGC GATACCTGAA

                    ....|....| ..******.| ********** ....|....| ....|....| ....|....| ....|....|
                             150       160       170       180       190       200       210
A. inc. PR17-594    CGTTCGCCGT TTT-----TT ---------- AAAAACGGTG TCGTTTGAAA TGATATGCAA GTTTCGGAGG
A. inc. PR16-371    CGTTCGCCGT TTT-----TT ---------- AAAAACGGTG TCGTTTGAAA TGATATGCAA GTTTCGGAGG
A. inc. PR16-374    CGTTCGCCGT TTT-----TT ---------- AAAAACGGTG TCGTTTGAAA TGATATGCAA GTTTCGGAGG
A. virlai [Arg]     CGTTCGCCGT TTACGCGCTT CCGAGTGTCG AAAAACGGTG TCGTTTGAAA TGATATGCAA GTTTCGGAGG
A. virlai [Mex]     CGTTCGCCGT TTACGCGCTT CCGAGTGTCG AAAAACGGTG TCGTTTGAAA TGATATGCAA GTTTCGGAGG

                    .*.*|....| ..*.|....* ********** ...***...| ...*|....| ....|.*..* ..***....|
                             220       230       240       250       260       270       280
A. inc. PR17-594    A-AATCGCTG ACCGAGAAAT TGAGTACG-- AACACAAGCT TGT-CTCGAA TTCTCTCTCG TGTTGAGCGA
A. inc. PR16-371    A-AATCGCTG ACCGAGAAAT TGAGTACGAA AACACAAGCT TGTCCTCGAA TTCTCTCTCG TGTTAAGCGA
A. inc. PR16-374    A-AATCGCTG ACCGAGAAAT TGAGTACGAA AACACAAGCT TGTCCTCGAA TTCTCTCTCG TGTTAAGCGA
A. virlai [Arg]     ATACTCGCTG ACGGAGAAA- ---------A AACTTGAGCT TGT-CTCGAA TTCTCTTTCA TG---AGCGA
A. virlai [Mex]     ATACTCGCTG ACGGAGAAA- ---------A AACTTGAGCT TGT-CTCGAA TTCTCTTTCG TG---AGCGA

                    *.*.|*...* ***.|....| ....|....| .********* ***.*....| ....|....| ....|....|
                             290       300       310       320       330       340       350
A. inc. PR17-594    TAACGCCGCG GACGACGCGC GTGTGCAGGT TT-------- --TTTTACCA TGCGAATACA CGCGAATGCG
A. inc. PR16-371    TAACGCCGCG GACGACGCGC GTGTGCAGGT TT-------- --TTTTACCA TGCGAATACA CGCGAATGCG
A. inc. PR16-374    TAACGCCGCG GACGACGCGC GTGTGCAGGT TT-------- --TTTTACCA TGCGAATACA CGCGAATGCG
A. virlai [Arg]     CAGCGTCGCA ATTGACGCGC GTGTGCAGGT TCGGTTTAAC TACTGTACCA TGCGAATACA CGCGAATGCG
A. virlai [Mex]     CAGCGTCGCA ATTGACGCGC GTGTGCAGGT TCGGTTTAAC TACTGTACCA TGCGAATACA CGCGAATGCG

                    ....|....| ....|....| ....|....| .**.|....| ....|....| ....|...*| .*****.*.|
                             360       370       380       390       400       410       420
A. inc. PR17-594    TCGGATGCAT TGCGTTCTCT TCCCGGAGCA T--AACAACG TGTCCGTGAG CTTTCTTGCG T-----TTAT
A. inc. PR16-371    TCGGATGCAT TGCGTTCTCT TCCCGGAGCA T--AACAACG TGTCCGTGAG CTTTCTTGCG T-----TTAT
A. inc. PR16-374    TCGGATGCAT TGCGTTCTCT TCCCGGAGCA T--AACAACG TGTCCGTGAG CTTTCTTGCG T-----TTAT
A. virlai [Arg]     TCGAATGCAT TGCGTTCTCT TCCCGGAGCA TACAACAACG TGTCCGTGAG CTTTCTTGTG TATTTTTGAT
A. virlai [Mex]     TCGAATGCAT TGCGTTCTCT TCCCGGAGCA TACAACAACG TGTCCGTGAG CTTTCTTGTG TATTTTTGAT

                    *...*...*| ....|....| ....*.*..| ....|....| ....|....| ....|....| ....|....|
                             430       440       450       460       470       480       490
A. inc. PR17-594    GCGCGATTAA GCGGACCGAT GTTAGACAGC ACTCCGAATG GGACAGAGAG TAGGCGTTGT CGTTATCGAG
A. inc. PR16-371    GCGCGATTAA GCGGACCGAT GTTAGACAGC ACTCCGAATG GGACAGAGAG TAGGCGTTGT CGTTATCGAG
A. inc. PR16-374    GCGCGATTAA GCGGACCGAT GTTAGACAGC ACTCCGAATG GGACAGAGAG TAGGCGTTGT CGTTATCGAG
A. virlai [Arg]     ACGCAATTGA GCGGACCGAT GTTA-ATAGC ACTCCGAATG GGACAGAGAG TAGGCGTTGT CGTTTTCGAG
A. virlai [Mex]     ACGCAATTGA GCGGACCGAT GTTA-ATAGC ACTCCGAATG GGACAGAGAG TAGGCGTTGT CGTTTTCGAG

                    ....|...** ...*|*...| *.***....| .**.|**..| ....|....| ....|..*** ....|....*
                             500       510       520       530       540       550       560
A. inc. PR17-594    AGGGAGAGAC ACTACCGTTT GTAC-TTTTC TCACGTTGGC ACCTTCCTTG TACAACGAGA CTTGCTTTTG
A. inc. PR16-371    AGGGAGAGAC ACTACCGTTT TTACTTTTTC TCACGTTGGC ACCTTCCTTG TACAACGAGA CTTGCTTTTG
A. inc. PR16-374    AGGGAGAGAC ACTACCGTTT TTACTTTTTC TCACGTTGGC ACCTTCCTTG TACAACGAGA CTTGCTTTTG
A. virlai [Arg]     AGGGAGAG-- ACTGCTGTTT GT-TATTTTC TTGCGAAGGC ACCTTCCTTG TACAACGATG CTTGCTTTTA
A. virlai [Mex]     AGGGAGAG-- ACTGCTGTTT GT-TATTTTC TTGCGAAGGC ACCTTCCTTG TACAACGRTR CTTGCTTTTA

                    ..*.*...** ....*..**| **.*|.**** ..*.*.*.** ....|.*..| ....*....| ....|....|
                             570       580       590       600       610       620       630
A. inc. PR17-594    TTAATATTCT TTTTTTT--C TAAAAA--GA GATGTTGACA AAAAAATTTT TTATTATACG ACGACCTCAG
A. inc. PR16-371    TTAATATTCT TTTTTTTTTC TAAAAAAAGA GATGTTGACA AAAAAATTTT TTATTATACG ACGACCTCAG
A. inc. PR16-374    TTAATATTCT TTTTTTTTTC TAAAAA--AA GATGTTGACA AAAAAATTTT TTATTATACG ACGACCTCAG
A. virlai [Arg]     TT-ACATTTC TTTTCTTTAC GGAGAATTGT GAAGGTAATG AAAAAA-TTT TTATCATACG ACGACCTCAG
A. virlai [Mex]     TT-ACATTTC TTTTCTTTAC GGAGAATTGT GAAGGTAATG AAAAAA-TTT TTATCATACG ACGACCTCAG

                    ....|....| ....|....| ....|...
                             640       650
A. inc. PR17-594    AGTAGGCGAG AATACCCGCT GAATTTAA
A. inc. PR16-371    AGTAGGCGAG AATACCCGCT GAATTTAA
A. inc. PR16-374    AGTAGGCGAG AATACCCGCT GAATTTAA
A. virlai [Arg]     AGTAGGCGAG AATACCCGCT GAATTTAA
A. virlai [Mex]     AGTAGGCGAG AATACCCGCT GAATTTAA
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Argentina, with consistent nucleotide substitutions at three positions (120, 121, and 270 in the aligned matrix) and "partial" differences at two further positions (adenosine and guanine were characteristic of the aligned matrix positions 548 and 550, respectively, in Argentinian specimens, but were ambiguous [both A or G] in four of the five Mexican specimens; Table 3). Sequences of the three other congeneric taxa confirmed them as distinct species, with multiple interspecific indels and substitutions (*A. avalae*, 662 bp; *A. fennicus*, 645 bp; *A. turpanicus*, 563 bp; see GenBank accessions). *Anagrus turpanicus* is a sister species to *A. virlai* (Fig 4), and that is also corroborated by their morphological similarity; however, to truly test that apparent sister relationship, more loci (particularly nuclear ones) and more specimens would need to be analyzed.

Discussion

This study highlights the effectiveness of simple molecular techniques for aiding the diagnoses of cryptic species. The molecular results corroborated very well with those of the morphometric analysis resulting in the identification of *A. virlai* as a separate entity from *A. incarnatus*. Although crossing experiments may be considered the "gold standard" for drawing species boundaries (under the biological species concept), in their absence, the corroborating genetic discrimination (genotypic clustering; Mallet 1995) provided serves as a good proxy. Separation of *A. virlai* from *A. incarnatus* makes sense from the point of view of the likely co-evolution of *A. virlai* with its major host, the corn leafhopper *D. maidis*, and also the plant hosts of that leafhopper, *Zea* spp., all of which are definitely New World in origin. Thus, *A. virlai* is quite likely to occur throughout the distribution range of its major leafhopper host, *D. maidis*. According to Palomera *et al* (2012), no genetic variation among Mexican and Argentinian populations of *D. maidis* was found in the nuclear ribosomal ITS2 region whereas a low genetic variation was detected in the mitochondrial COI region. So, similar to the conclusions made by Palomera *et al* (2012) for *D. maidis*, our results could also be explained by the short evolutionary time scale, since both cultivated maize and the corn leafhopper, host of *A. virlai*, moved throughout the Americas only in recent millennia. The small amounts of variation in the COI and ITS2 sequences across our specimens of *A. virlai* (and across specimens of *A. incarnatus*) are well within accepted intraspecific tolerances, but may provide some suggestion of population structure across its geographic range (Fig 4, Table 3). This could be tested/confirmed with a larger sample size.

In the maize-producing countries of the Americas where *D. maidis* is an agricultural pest and a known vector of plant diseases that inflict serious damage to corn crop, one of the possible solutions is enhancement of its biological control. Further refinement in the taxonomy of *A. virlai* populations could be of particular importance.

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