

Parasitism of the “Fuller’s rose weevil” *Naupactus cervinus* by *Microctonus* sp. in Argentina

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Abstract We report the occurrence of an unidentified species of the wasp *Microctonus* Wesmael (Hymenoptera: Braconidae) parasitizing adults of the Fuller’s rose weevil *Naupactus cervinus* (Boheman) (Coleoptera: Curculionidae), a widespread pest of economically important crops included in the South American tribe Naupactini. Cytochrome *c* oxidase subunit I-based phylogenetic analysis indicates that the parasitoid is closely related to *Microctonus hyperodae* Loan. Their first instar larvae show slight morphological differences with this species. Superparasitism by first instar larvae occurred at low frequency. Some teratocytes were observed. *Microctonus* sp. and its host were infected with different

strains of the reproductive parasite *Wolbachia pipientis* Hertig (Rickettsiales: Rickettsiaceae), although the bacterial lineage harbored by the wasp coincides with that infecting most parthenogenetic Naupactini. This multipartite association (weevil bearing both *Wolbachia* and *Microctonus*, and *Microctonus* bearing *Wolbachia*) opens challenging perspectives for future research on biological interactions and biological control.

Keywords Naupactini · Braconidae · Parasitoid · *Microctonus* · *Wolbachia* · Thelytoky

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Introduction

The knowledge of natural enemies of weevils is crucial to develop biological control strategies (Lanteri et al. 1998). The available information is limited to pathogens and parasites of some introduced species that cause important damage to different crops and plantations, e.g. the “cotton boll weevil” *Anthonomus grandis* Boheman in the USA, the “*Eucalyptus* weevil” of the genus *Gonipterus* in Europe and the Americas, and the “Argentine stem weevil” *Listronotus bonariensis* (Kuschel) in New Zealand (Lanteri et al. 2002). Potential changes in geographic ranges and relative abundances of insect pests like *Naupactus cervinus*, wrought by modern production practices, crop expansion and climate change emphasize the

need to design sustainable pest management programs. Biological control of weevils harmful for alfalfa and other cultivated Fabaceae (e.g. *Hypera postica* and *Sitona discoideus*) have been applied with success in the USA, Europe and New Zealand using braconid species of the genus *Microctonus* (Goldson et al. 1993; Barratt 2004). The finding of a *Microctonus* species parasitizing the worldwide distributed “Fuller’s rose weevil” opens a new perspective for its control in the countries where it has been introduced.

Natural enemies of weevils include bacteria, protozoans, fungi, mites and insects (Lanteri et al. 1998). Insect parasitoids are of major importance due to their diversity and potential as biological control agents. They exploit all developmental stages of the weevil, which is unable to complete its life-cycle and/or dies before reproduction. Most weevil parasitoids belong to the hymenopteran superfamilies Chalcidoidea and Ichneumonoidea (especially Braconidae), and to the dipteran family Tachinidae (Lanteri et al. 1998).

In Argentina, larvae and adults of weevil pests have been found parasitized by over 15 braconid species (Lanteri et al. 1998). Among these, the South American species *Microctonus hyperodae* Loan (Braconidae: Euphorinae) was imported into New Zealand in 1991 as a biocontrol agent of adult *L. bonariensis* (Goldson et al. 1993). Another species of this genus, the Palaearctic *Microctonus aethiopoulos* Loan, was also released in New Zealand in 1982 for the biocontrol of the Mediterranean weevil *S. discoideus* Gyllenhall (Barratt 2004). Wasps of the latter species from Ireland were used for the control of the weevil *Sitona lepidus* Gyllenhall in New Zealand (McNeill et al. 2006). These *Microctonus* species are solitary endoparasitoids with similar morphology and life cycle (Loan and Holdaway 1961). Occasional superparasitism has been noted, with supernumeraries being eliminated in the first larval stage. Adult weevils usually die soon after the mature parasitoid larva emerges. Then, the larva spins a cocoon close to the soil surface and roots where the pupa develops, and the adult emerges between ten and 19 days (average 15.6 days). *Microctonus hyperodae* reproduces by thelytoky (males absent or extremely rare) and apomixis (parthenogenesis without meiosis) (Iline and Phillips 2004), *M. aethiopoulos* is arrhenotokous in most European countries (unfertilized eggs produce males and fertilized eggs produce females) (McNeill et al. 1993), and the biotype of *M. aethiopoulos* in

Ireland is thelytokous and gregarious (McNeill et al. 2006). Moreover, *M. hyperodae* is monophagous to oligophagous (Goldson et al. 1992) whereas *M. aethiopoulos* is polyphagous (Goldson et al. 2005). There is also an endemic *Microctonus* species in New Zealand, *Microctonus zealandicus* Shaw, which is gregarious (2–6 larvae per host capable of reaching the pupal stage), arrhenotokous, and specific to the native broad-nosed weevil *Irenimus aequalis* (Broun) (McNeill et al. 1993; Shaw and Huddleston 1991).

In the present contribution we study a parasitoid of adult females of the “Fuller’s rose weevil” *N. cervinus* (Boheman) (Curculionidae: Entiminae: Naupactini), a parthenogenetic species native to South America and distributed worldwide due to its association with crops of economic importance, such as alfalfa, citrus and ornamental plants (Rodriguero et al. 2010a, b). Since *N. cervinus* is infected with the reproductive parasite *Wolbachia pipientis* Hertig (hereafter *Wolbachia*) (Rodriguero et al. 2010a), it is interesting to check if the parasitoid could be a potential vehicle for the horizontal transfer of this bacterium (Werren et al. 1995), thus contributing to its pandemic distribution.

Materials and methods

Sample collection

Adult females of *N. cervinus* were collected from 18 locations in Argentina and Brazil (N = 92) using sweeping nets during summer 2004–2013 (Table S1). They were dissected under a stereo microscope and examined for the presence of parasitoids. All larvae were preserved in 100 % ethanol and stored at 4 °C.

Molecular identification

PCR and sequencing

The parasitoid was identified by sequencing a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (COI), the standard DNA barcode for animal species (Hebert et al. 2003a, b). Total genomic DNA was extracted from two bulks of four larvae each using the REDExtract-N-Amp Tissue kit (Sigma-Aldrich). A segment of ca. 700 bp of the COI was amplified using the specific primers S1718 and A2442 (Rodriguero et al. 2010b). Amplification was carried out in a

50 µl-volume reaction with 50–100 ng of DNA used as template, 0.5 µM of each primer, 0.1 mM of each dNTP (Promega), 3.0 mM MgCl₂, 1.0 unit of Taq polymerase and 1× reaction buffer (Invitrogen). The reactions were performed in a Verity thermal cycler (Applied Biosystems), under the conditions described by Rodriguez et al. (2010b). PCR products were purified with a QIAquick Gel Extraction Kit (Qiagen Inc.). DNA was sequenced using a 3130-XL Automatic Sequencer (Applied Biosystems). Standard chromatographic curves of forward and reverse sequences were edited with the program BIOEDIT v. 7.0.5.3 (Hall 1999).

Phylogenetic analysis

For parasitoid identification we followed the procedure of Sasakawa et al. (2011). First, we identified the family to which this parasitoid belongs through comparison of the sequence obtained with previously published sequence data using BLASTn sequence analysis (Altschul et al. 1990, 1997). Second, we accomplished a genus-level analysis including COI sequences of our samples and other members of Braconidae. Third, we compared genetic distances of COI sequences between our samples and putative congeneric species and tested whether our genus-level identification was compatible in terms of genetic variability.

Cytochrome *c* oxidase subunit I sequences of Hymenoptera were retrieved from GenBank (NCBI) (see accession numbers in Fig. 2). The Clustal W software v. 2.0S was used for sequence alignments (Thompson et al. 1994), with default parameter settings. The best molecular evolution model was chosen on the basis of the Akaike information criterion (Posada and Buckley 2004) using MRMODELTEST software v. 2.2 (Nylander 2004).

Phylogenetic analysis included 50 sequences of five genera and one representative of Ichneumonidae was used as outgroup. Our complete dataset included 676 aligned nucleotide positions. The GTR + I + G model was selected as the best fit model of nucleotide substitution.

We applied Bayesian inference based on Metropolis coupled Markov chain Monte Carlo algorithm implemented in MRBAYES v. 3.2.2 (Ronquist et al. 2012). Two independent analyses were run with a random starting tree over 2,000,000 generations with a

sample frequency of 500. The tree space was explored using one cold and three incrementally heated chains, with temperature set to 0.20. We assessed stationarity of the cold Markov chain for all MRBAYES analyses through the standard deviation of the split frequencies. All posterior samples of a run prior to the burn-in point (at 25 % of sampled topologies) were discarded. Remaining trees were used to obtain a 50 % majority-rule consensus tree with mean branch length estimates. Node support was assessed by posterior probability (Huelsenbeck and Ronquist 2001).

Genetic distances between genera and between congeneric species were estimated by pairwise comparisons using MEGA v. 6 (Tamura et al. 2013). Several sequences were used for each species, depending on data availability.

Morphological identification

First instar larvae were cleared in 10 % KOH for 24 h, slide-mounted in Canada Balsam and examined under a light microscope LeitzWetzlar SM/LUX. Shape and size of the head, capsule and mandibles and the pattern of hypopharyngeal sclerotization are the most reliable characters for specific identification of first instar larvae of *Microctonus* (Fuester 1970). However, parasitoids usually need to be reared to the adult stage for species identification (Phillips and Emberson 1999). Mature hymenopteran larvae were not described because they do not allow taxonomic identification beyond the order level (Sasakawa et al. 2011). Both first instar and mature larvae were photographed using a Leica DFC295 digital camera attached to a stereo microscope Leica S8 APO. Digital images were mounted using free software Combine ZM (Hadley 2011) and enhanced using Photoshop.

Wolbachia detection

Wolbachia infection was surveyed through amplification of genes 16S rDNA and *gatB*, using the primers designed by O’Neill et al. (1992) and Baldo et al. (2006) for the host and parasitoid, respectively. We randomly chose six individuals of *N. cervinus* from the locations where the parasitoid had been found to detect the occurrence of infection, as previously reported for many populations of this weevil (Rodriguero et al. 2010a, b). Thermal profiles and negative controls were

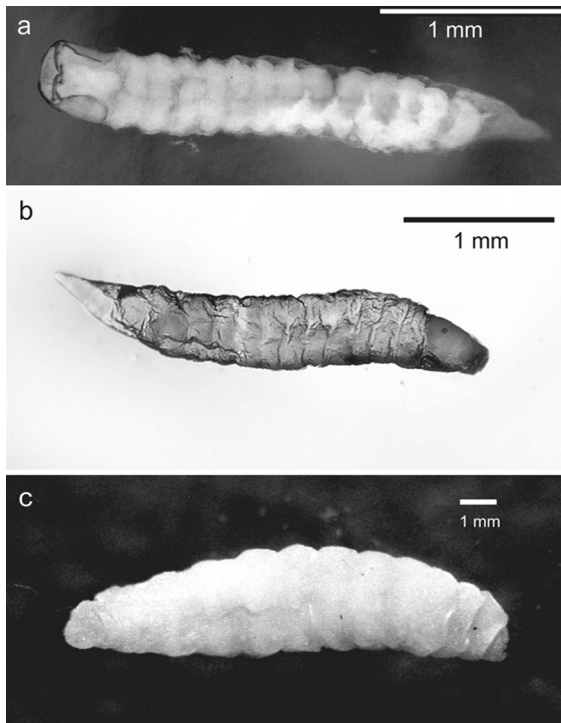


Fig. 1 Larva of *Microctonus* sp.: **a** first instar, ventral view, scale 1 mm; **b** first instar, lateral view, scale 1 mm; **c** mature larva, lateral view, scale 1 mm

performed as previously described (Rodriguero et al. 2010a).

Wolbachia strain in the parasitoid was characterized by full MLST. Amplification and sequencing of *coxA*, *fbpA*, *ftsZ*, *gatB* and *hcpA* fragments followed those of Baldo et al. (2006). Allele number per locus was assigned after comparison with the *Wolbachia* MLST database (<http://pubmlst.org/wolbachia>). This strain was characterized by the combination of the MLST numbers (i.e. allelic profile or ST).

Results

We dissected 92 *N. cervinus* adults collected from 18 localities of Argentina and Brazil, and found six first instar larvae parasitizing one weevil in La Paz (northwest of Entre Rios province, north-eastern Argentina) (Table S1; Fig. 1a, b). The percentage of parasitism in this population was 16.7 % (one of six weevils). First instar larvae had a mean length of 1.65 mm. In addition, 46 mature larvae emerged from 70 weevils collected in Tandil (southeast of Buenos

Aires province, central-eastern Argentina) while being transported to the laboratory, and 22 % of them pupated, although no emergence of adults was seen under lab conditions (Fig. 1c). The percentage of parasitism in this population was 65 %. Mature larvae had a mean length of 11.4 mm. Neither first instar nor mature larvae showed signs of host immune response, such as melanization. Some teratocytes were observed in the body cavity of *N. cervinus*.

Molecular identification of parasitoids

The larvae from *N. cervinus* share almost identical mitochondrial sequences, except for two ambiguous bases: A/G and A/G at positions 452 and 473, respectively. Because of the low number of unambiguous positions, we chose a consensus sequence to define their phylogenetic position within Hymenoptera (G at positions 452 and 473 based on the electropherogram peak heights, GenBank Accession Number KJ158158).

BLASTn homology search indicated that the COI sequence of the *N. cervinus* parasitoid shared 82–85 % nucleotide identity with the COI sequences of several Braconidae, including genera such as *Microctonus* (e.g. GenBank accession no. KJ158158.1, E-value = 0.0), *Opius* (e.g. GenBank accession no. KF474863.1 E-value = $1e-111$), *Adialytus* (e.g. GenBank accession no. HQ724539.1 E-value = $3e-118$), *Binodoxis* (e.g. GenBank accession no. FJ798201 E-value = $3e-118$) and *Chelonus* (GenBank accession no. HQ106889.1 E-value = $3e-118$). Thus, this analysis reveals that the parasitoid of *N. cervinus* belongs to Braconidae. Consequently, the subsequent genus-level analysis includes five genera of Braconidae.

The studied parasitoid falls within the genus *Microctonus*, with high nodal support (Fig. 2). The tree shows that it is more closely related to *M. hyperodae* than any other *Microctonus* species. Genetic distances between the parasitoid and other *Microctonus* spp. range between 13 and 17 % (Table 1), which is similar to those among remaining *Microctonus* species (e.g. 13–16 % between *M. zealandicus* and other *Microctonus* spp.). However, the parasitoid and other braconid genera are separated by a wider range of distance (20–26 %).

Morphological identification of parasitoids

Based on the molecular identification of the first instar larvae parasitizing *N. cervinus*, we described the head

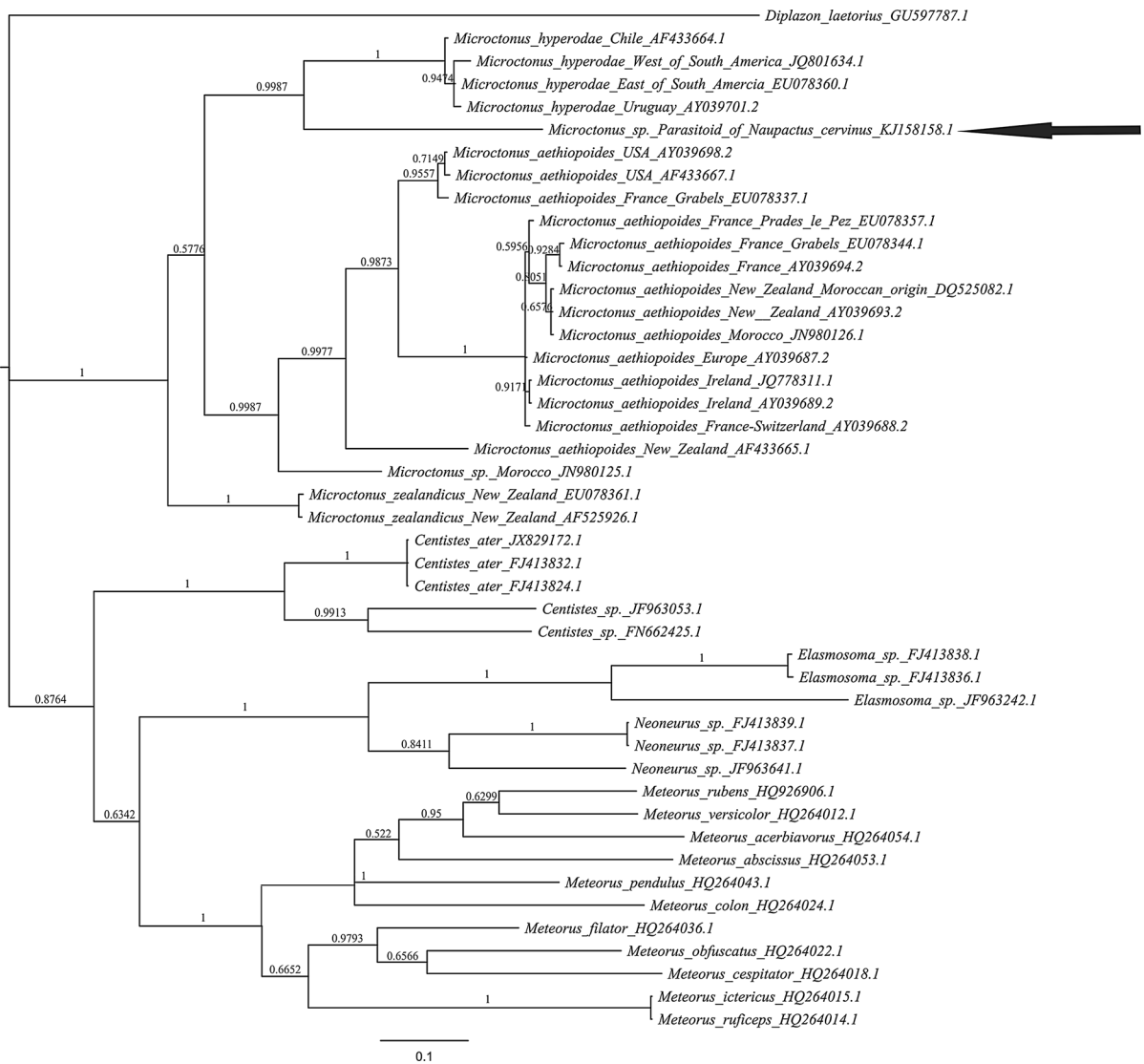


Fig. 2 Phylogenetic position within Braconidae of parasitoid larvae from *Naupactus cervinus* adults on the basis of COI sequences indicated with an arrow. Numbers above the branches

indicate node support (posterior probability). The scale bar represents the number of substitutions per site

capsule, mandibles and hypopharynx of this species of the genus *Microctonus*.

Diagnosis of *Microctonus* sp.

First instar larva 1.0–2.1 mm long, of caudate-mandibulate type (Fig. 1a). Head capsule pale-yellow, about $0.9 \times$ as long as wide, with sides nearly straight (Fig. 1b). Mandibles large, with lateral walls basally thickened. Length/width ratio of mandibles greater than 2.5 (Fig. 3). Hypopharynx parallel-sided, not

tapering from front to rear. Hypopharyngeal sclerites poorly developed (Fig. 3). Measurements taken from slide-mounted larva (μm): total body length 1,645; mandible length 100–106; mandible width 40–42; head capsule length 232; head capsule width 264.

Comparison with other *Microctonus* larvae

The first instar larva described herein is similar to that of *M. hyperodae*, except for the size of the head capsule (shorter in *M. hyperodae*: $0.82 \times$ as long as wide) and the

Table 1 Pairwise genetic distances between species of the genus *Microctonus* and other genera of Braconidae

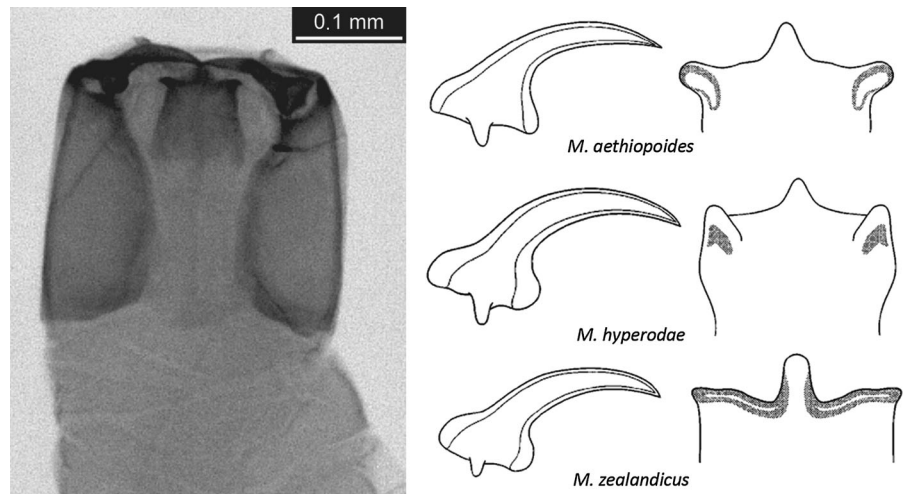
Taxa	Genetic distances								
Larva from <i>N. cervinus</i>									
<i>M. hyperodae</i> ^a East	0.139								
<i>M. hyperodae</i> ^a West	0.145	0.006							
<i>M. zealandicus</i>	0.160	0.136	0.140						
<i>M. aethiopoides</i> from <i>Hypera</i> sp. ^b	0.160	0.147	0.153	0.146					
<i>M. aethiopoides</i> from <i>Sitona</i> sp. ^b	0.171	0.159	0.163	0.165	0.075				
<i>Centistes</i>	0.224	0.223	0.225	0.211	0.221	0.227			
<i>Elasmosoma</i>	0.258	0.249	0.251	0.243	0.256	0.257	0.242		
<i>Meteorus</i>	0.206	0.195	0.199	0.198	0.186	0.201	0.225	0.243	
<i>Neoneurus</i>	0.243	0.219	0.221	0.225	0.227	0.236	0.241	0.215	0.206

Biotypes of *Microctonus* species are distinguished when sequences were available

^a East and West biotypes of *M. hyperodae* after Iline and Phillips (2004)

^b Biotypes of *M. aethiopoides* after their host weevil, Vink et al. (2003)

Fig. 3 Left detail of head capsule (mandibles and hypopharynx) of first instar larva of *Microctonus* sp., ventral view. Right Drawings of mandibles and hypopharynx of other *Microctonus* species taken from Phillips and Emberson (1999), with permission



length/width ratio of mandibles (smaller in *M. hyperodae*: 2.1–2.5). When viewed ventrally, after being compressed by the microscope slide coverslip, the basal third of the mandible is similar to that of *M. hyperodae* and *M. zealandicus*, namely flared or vase-like in shape with a distinct sinuation on the outer margin (Fig. 3). However, it differs from the latter two species and resembles *M. aethiopoides* in having poorly developed hypopharyngeal sclerites, which protrude laterally from the hypopharynx (Fig. 3). The hypopharynx of the studied *Microctonus* sp. differs from that of *M. zealandicus* in the lack of a very distinctive band of sclerotization, which is effaced medially and extends across the anterior margin (Fig. 3).

Molecular detection and identification of *Wolbachia*

We positively diagnosed *Wolbachia* infection for the parasitoids of *N. cervinus* and hosts from La Paz (N = 6) and Tandil (N = 6). The sequencing of MLST genes of the parasitoid strain yields alleles 14, 181 and 9 for loci *coxA*, *fbpA* and *gatB*, respectively. Unfortunately, the *ftsZ* and *hcpA* genes could not be amplified. This partial sequence type (ST) does not coincide with the one infecting *N. cervinus* (ST = 14-9-127, strain wNau5), but closely resembles to the *Wolbachia* strain from most of the parthenogenetic Naupactini weevils (ST = 14-181-9, strain

wNau1). It belongs to *Wolbachia* Supergroup B (Rodríguez et al. 2010a).

Discussion

This is the first report of a parasitoid of the genus *Microctonus*, family Braconidae, infesting the weevil pest *N. cervinus*. At present, the only known parasitoids of this species are the braconid *Centistes* sp., a parasitoid of the larval stage (Artigas 1994), and the platygastriid *Fidiobia citri* (Nixon), an egg parasitoid (Morse et al. 1988). The presence of *Centistes* sp. has also been reported for another weevil pest native to South America, the “fruit weevil” *Naupactus xanthographus* (Germar) (Artigas 1994; Lanteri et al. 1998).

The genus-level analysis indicated that the parasitoid recovered from adult *N. cervinus* clusters with *M. hyperodae* with a high support. We have no hesitation to assign the species to the genus *Microctonus* since it is included within the *Microctonus* clade.

The following four species of *Microctonus* are native to South America: *M. hyperodae*; *Microctonus audax* Muesebeck from Argentina, parasitoid of *Listroderes* sp.; *Microctonus berryi* Muesebeck from Central Chile, parasitoid of Chrysomelidae; and *Microctonus brasiliensis* (Szepligeti) from Brazil, of unknown host (Loan and Lloyd 1974). *M. hyperodae* is the best known and most widespread species of the genus in South America (Loan and Lloyd 1974). It has two biotypes differentiated by genetic markers based on variation in malate dehydrogenase allozymes, originating from east (Argentina, Brazil and Uruguay) and west (Chile) of the Andes mountains (Iline and Phillips 2004; Winder et al. 2005).

Branch lengths suggest high divergence between the studied *Microctonus* sp. and *M. hyperodae* biotypes. In fact, genetic divergence between these entities are similar to values for interspecific comparisons between *Microctonus* species (Sasakawa et al. 2011). In addition, genetic distances between the parasitoid of *N. cervinus* and other *Microctonus* species are smaller than those between the former and species of other genera. The species studied here is distinguished from *M. hyperodae* by the following criteria: (1) Morphological characters at larval level are compatible with species differentiation, although the species status should be confirmed with the

taxonomic study of adult specimens. On the opposite, the two highly divergent biotypes of *M. hyperodae* are morphologically identical (Phillips and Emberson 1999). (2) *M. hyperodae* seems to be a species-specific parasitoid of *L. bonariensis* since it is its only weevil host so far reported for South America. Host-preference studies carried out in New Zealand revealed that *M. hyperodae* wasps preferred to lay eggs in *L. bonariensis*, only 3 % of the parasitoids emerged from other weevil species, and the proportion of melanized larvae (signs of a host immune response) was over 40 % in the alternative hosts and about 8 % in *L. bonariensis* (Barratt 2004). (3) *M. hyperodae* and *N. cervinus* are partially sympatric in South America. However, the latter is more widespread in subtropical areas of Brazil and *L. bonariensis* in arid environments of central and southern Argentina, including Patagonia. (4) Genetic divergences between the studied parasitoid and both *M. hyperodae* biotypes exceed the limits of intraspecific variation (<4.0 % for COI sequences, Danforth et al. 1998). Moreover, this divergence is higher than that observed between the two biotypes of *M. aethioides* (9 %), which is a consequence of the specialization of the wasps to different host weevils, although they are still considered a single species (Vink et al. 2003, 2012; Phillips et al. 2008).

Based on the current evidence and until more detailed studies are made (e.g. lab rearing of parasitoids to the adult stage for consistent identification of species), we assume that the studied *Microctonus* sp. is a new or an already known South American species that can only be identified on the basis of adult characters. The possible species could be *M. audax* or *M. brasiliensis*, whose geographic distributions are within the natural geographic range of *N. cervinus*. Phylogeny estimates from morphological and molecular data are congruent in the sense that both suggest a close relationship between the putative new species of *Microctonus* and *M. hyperodae*.

The finding of several first instar larvae of *Microctonus* sp. in a single female of *N. cervinus* may suggest superparasitism, but it is necessary to determine if only one larva outcompetes siblings within the host. Although *M. hyperodae* and *M. aethioides* are solitary endoparasitoids, they may initially be supernumerary, what has been proposed as a strategy to suppress host defences (Barratt and Johnstone 2001). Moreover, the number of supernumeraries is likely to

depend on host size. The available information precludes us from drawing firm conclusions about whether the studied *Microctonus* sp. is solitary as *M. hyperodae* and *M. aethiopoulos*, or gregarious as *M. zealandicus*.

Some teratocytes were observed in the body cavity of *N. cervinus*. These cells, which are derived from the serosal membrane of parasitoid eggs, may be nutritive, immunosuppressive, or secretory and can be involved in regulating host development (Dahlman and Vinson 1993). In Euphorinae they are thought to have a clear trophic role, and are extensively fed upon by middle-instar larvae, whose mandibles are reduced or absent (Shaw and Huddleston 1991). Further studies are needed to elucidate the function of teratocytes for the parasitoid of *N. cervinus*.

The probability of success as a biological control agent in exotic locations is greater when parasitism rates are high in the native range (McNeill et al. 2006). On this account, the population of Tandil has good potential for controlling *N. cervinus* in countries where it has been accidentally introduced, such as Australia, New Zealand or the USA. Further research is needed on this issue.

Parasitoids have been suggested as one of the potential vehicles of *Wolbachia* between species. This possible route of inter-taxon transfer has received both supporting (e.g. Werren et al. 1995, i.e. highly similar *Wolbachia* strains in both host and parasitoid) or refuting (e.g. West et al. 1998, i.e. highly dissimilar *Wolbachia* strains in both host and parasitoid) evidence. In the case under study, the host and parasitoid were found to have different strains, but the strain isolated from the wasp, wNau1, infects most of the parthenogenetic Naupactini species studied so far (Rodríguez et al. 2010a). Although this result makes horizontal transfer of *Wolbachia* between *Microctonus* sp. and *N. cervinus* unlikely, such transmission between the studied *Microctonus* sp. and other Naupactini hosts cannot be ruled out. It may parasitize more than one Naupactini species (same as *Centistes* sp.), thus spreading the strain wNau1 among species such as *Aramigus conirostris* (Hustache) or *Atrichonotus taeniatus* Berg. The infection of *N. cervinus* by the strain wNau5 could have been acquired from another parasitoid or through other means used by *Wolbachia* to overcome interspecific barriers (e.g. Kittayapong et al. 2003).

There is a noteworthy relationship among hosts, parasitoids, environment and *Wolbachia* infection. Broad-nosed weevils of the subfamilies Entiminae and Cyclominae are among the main hosts for euphorine braconids, such as *M. hyperodae*, *M. aethiopoulos* and *M. zealandicus* (Shaw 1988). Some of them are flightless species associated with native or exotic grasslands, pastures and alfalfa, and reproduce by parthenogenesis (Lanteri and Normark 1995; Marvaldi et al. 2012), same as their parasitoids. This kind of reproduction facilitates the dispersal and colonization of new areas outside the species' natural range (Guzmán et al. 2012; Lanteri et al. 2013). In the context of the relationship among weevil pests, braconid parasitoids and *Wolbachia* infection, our finding of a *Microctonus* species parasitizing the parthenogenetic species *N. cervinus* opens challenging perspectives for future research on biological interactions. Their better understanding is crucial for a more effective biological control of the "Fuller's rose weevil" and other weevil pests.

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