Syst Parasitol (2013) 86:43–51
DOI 10.1007/s11230-013-9428-5

Sequence analysis of the internal transcribed spacer 2 (ITS2) from *Philornis seguyi* (García, 1952) and *Philornis torquans* (Nielsen, 1913) (Diptera: Muscidae)

Lucas D. Monje · Martín Quiroga · Darío Manzoli · Márcia S. Couri · Leonardo Silvestri · José M. Venzal · Pablo Cuervo · Pablo M. Beldomenico

Received: 16 January 2013 / Accepted: 5 June 2013

Abstract *Philornis* Meinert, 1890 (Diptera: Muscidae) is a genus of Neotropical dipterans that parasitise birds. The currently used external morphological characters to distinguish between species within this genus present some limitations. We used the second internal transcribed spacer region (ITS2) of the rRNA gene as a molecular marker to differentiate adult specimens of *Philornis* identified morphologically as *Philornis torquans* and *Philornis seguyi* from different localities. Specimens identified as *P. seguyi* from Magdalena (Buenos Aires Province) showed an ITS2 sequence different from that for *P. torquans*, whereas all other specimens of *P. seguyi* had sequences identical to those for *P. torquans*. These findings do not necessarily confirm that specimens from Magdalena indeed belong to *P. seguyi*, nor that *P. seguyi* is a valid species. Instead, they alert us about the potential for species misidentification when using morphological characters alone. The use of molecular approaches to aid the identification of *Philornis* spp. will shed light on the systematics of this group. *P. torquans* is reported for the first time in Mendoza Province and Uruguay.

Keywords Avian ectoparasite · *Philornis torquans* · *Philornis seguyi* · *Philornis downsi* · Internal transcribed spacer 2

L. D. Monje (✉) · M. Quiroga · D. Manzoli · L. Silvestri · P. M. Beldomenico
Laboratorio de Ecología de Enfermedades, Instituto de Ciencias Veterinarias del Litoral (ICiVet Litoral), Universidad Nacional del Litoral—Consejo Nacional de Investigaciones Científicas y Tecnológicas (UNL—CONICET), Santa Fe, Argentina
e-mail: lmonje@fcv.unl.edu.ar

M. S. Couri
Museu Nacional, Universidade Federal do Rio de Janeiro, Quinta da Boa Vista, Sao Cristovao, Rio de Janeiro, Brazil

J. M. Venzal
Departamento de Parasitología Veterinaria, Facultad de Veterinaria, Universidad de la República, Regional Norte, Salto, Uruguay

P. Cuervo
Centro de Investigación en Parasitología Regional, Facultad de Ciencias Veterinarias y Ambientales, Universidad Juan A. Maza, Mendoza, Argentina

Introduction

Philornis Meinert, 1890 (Diptera: Muscidae) is a genus of Neotropical dipterans that parasitise birds (Couri, 1999; Dudaniec & Kleindorfer, 2006; Antoniazzi et al., 2011). A few species of the genus have larvae with coprophagous trophic behaviour whereas the larvae of at least 20 species have been confirmed to be obligatory parasites of a broad diversity of birds in the New World (Couri, 1999) that may cause detrimental effects on their fitness and survival (Galligan & Kleindorfer, 2009; Fessl et al., 2010; Norris et al., 2010; Antoniazzi et al., 2011; Deem et al., 2012).

Eight species of *Philornis* have been recorded in Argentina: *P. angustifrons* (Loew, 1861), *P. blanchardi* Garcia, 1952, *P. nielseni* Dodge, 1968, *P. pici* (Macquart, 1854), *P. seguyi* Garcia, 1952, *P. torquans* (Nielsen, 1913), *P. umanani* Garcia, 1952, and *P. downsi* Dodge & Aitken, 1968 (see Couri et al., 2009; Silvestri et al., 2011 and references therein). According to recent reviews, only four of these are recognised as valid (Couri et al., 2009; Silvestri et al., 2011): *P. torquans, P. seguyi, P. blanchardi* and *P. downsi*. Recently, specimens of *P. torquans* and *P. seguyi* have been identified in Santa Fe and Buenos Aires provinces (Argentina) and *P. downsi* has been recorded in Chaco Province (Argentina) (Couri et al., 2005; Rabuffetti & Reboreda, 2007; Couri et al., 2009; Antoniazzi et al., 2011; Segura & Reboreda, 2011; Silvestri et al., 2011; Quiroga & Reboreda, 2012). *Philornis blanchardi* is only known from its original description based on a single specimen from Corrientes Province (see Garcia, 1952); however, the type material is apparently lost (Couri et al., 2009). The larvae of *P. downsi* are nest–dwelling and have semi-haematophagous habits, the behaviour of the larvae of *P. blanchardi* is unknown, whereas the larvae of *P. torquans* and *P. seguyi* are subcutaneous tissue feeders which, after penetrating host integument undergo a period of development and growth, and become established between the dermis and the superficial muscles, where they are easily observed (Texeira, 1999; Couri et al., 2007).

In Argentina, the geographical distributions of *P. torquans* and *P. seguyi* overlap (Couri et al., 2009). In addition, distinguishing these two species proved to be difficult using morphological characters when large series are analysed (reviewed in Couri et al., 2009). DNA analysis showed to be a useful tool that may help overcome this issue.

Accumulating evidence suggests that ITS2 of the rRNA gene is a good phylogenetic marker at the species or generic levels due to its many advantages, such as high information content in a relatively short region to amplify (Young & Coleman, 2004). Additionally, the ITS2 region has been widely used to differentiate mosquito and fly species (Thanwisai et al., 2006; Walton et al., 2007; LaRue et al., 2009; Paredes-Esquivel et al., 2009). In general, accurate identification of the parasite species is of vital importance since many traits of the host-parasite association depend on the parasite species involved. For example, coccidia of the genus *Eimeria* Schneider, 1875 largely differ in their virulence and could be classified into five groups: non pathogenic, slightly pathogenic, mildly pathogenic, highly pathogenic and species with "pathogenicity depending on the infective dose" (reviewed in Pakandl, 2009). This is something that should definitely be taken into account when dealing with species of *Philornis*, a genus that includes both generalist and specialist species (Lowenberg-Neto, 2008) that also exhibit different types of interactions with their hosts (Dudaniec & Kleindorfer, 2006). Empirical data support this notion since several studies report quite dissimilar effects of infections with *Philornis* spp. such as negative (Rabuffetti & Reboreda, 2007; Antoniazzi et al., 2011; Quiroga & Reboreda, 2012), slight (Nores, 1995) or none (Young, 1993).

In the present study, we use the ITS2 of the rRNA gene as a molecular marker to differentiate adult specimens identified as *P. torquans* and *P. seguyi* from Argentina and Uruguay. In order to confirm that the approach employed here is useful to distinguish species within the genus, we compared our results with the available ITS2 sequences for *Philornis downsi* obtained from specimens collected in Chaco Province (ARGcha) (Silvestri et al., 2011).

Materials and methods

Sample collection and morphological identification

Larvae, pupae and adults of *Philornis* spp. were collected in localities from three Argentinean provinces (Santa Fe, Buenos Aires and Mendoza) and in Uruguay (Table 1; Fig. 1). The third instar larvae and pupae were incubated at room temperature to obtain the adults. In the case of the larvae from Escobar city (ARGesc), adults were not obtained. All specimens were preserved in ethanol or silica gel according to the criteria of collection curators.

Adults were examined and identified to species in Museu Nacional (Rio de Janeiro) by Márcia Couri, Leonardo Silvestri and Martín Quiroga using the keys of Couri (1999), de Carvalho & Couri (2002) and Couri et al. (2009). This resulted in identification of *P. torquans* in the samples from Esperanza (ARGesp) and Uruguay (URUsal), and of *P. seguyi* in the

Table 1 Species of *Philornis* identified, their hosts and the ID labels of the DNA samples sequenced

Species (morphological identification)	Locality	Sample size/stage	Host	Source of material	DNA sample ID
Philornis torquans	Esperanza (Argentina)	40 adults	*Pitangus sulphuratus* Linnaeus (Tyrannidae); *Phacellodomus ruber* Vieillot (Furnariidae); *Phacellodomus sibilatrix* Sclater (Furnariidae)	Antoniazzi et al. (2011)	1-ARGesp; 3-ARGesp; 4-ARGesp; 20-ARGesp; 21-ARGesp
Philornis torquans	Salto (Uruguay)	13 adults	*Pitangus sulphuratus* (Tyrannidae)	This study	2-URUsal; 13-URUsal; 16-URUsal
Philornis seguyi	Santa Fe (Argentina)	315 adults	*Troglodytes aedon* Vieillot (Troglodytidae)	Quiroga & Reboreda (2012)	1-ARGstf; 3-ARGstf; 4-ARGstf; F3-ARGstf; F4-ARGstf; C22-ARGstf
Philornis seguyi	Magdalena (Argentina)	5 adults	*Molothrus bonariensis* Gmelin (Icteridae)	Couri et al. (2005)	17-ARGmgd; 22-ARGmgd; 24-ARGmgd
Philornis seguyi	La Paz department (Argentina)	3 adults	*Turdus amaurochalinus* Cabanis (Turdidae)	This study	10-ARGlpz; 11-ARGlpz; 12-ARGlpz
Philornis downsi	Chaco (Argentina)	6 adults	*Sicalis flaveola pelzelni* Sclater (Emberizidae)	Silvestri et al. (2011)	1-ARGcha; 2-ARGcha; 3-ARGcha
Unidentified larval *Philornis* spp.	Escobar (Argentina)	4 larvae	*Mimus saturninus* Linnaeus (Mimidae)	This study	1-ARGesc; 2-ARGesc

samples from Santa Fe (ARGstf), Magdalena (ARGmgd) and La Paz (ARGlpz). The specimens were also systematically examined, considering all diagnostic external morphological characters and the morphology of the genitalia.

Molecular characterisation

Two unidentified *Philornis* larvae and twenty three adult flies were processed and analysed (Table 1). Total genomic DNA was extracted from individual flies and larvae using the complete specimens. Single specimens were grounded in 450 µl of lysis buffer (10 mM Tris-Cl, 100 mM EDTA, 0.5% SDS, pH 8.0), and then proteinase K (final concentration 200 µg/ml) was added to the mixture. Samples were then incubated in 50 °C water bath overnight. After incubation, proteinase K was inactivated by boiling samples for 5 min. The following DNA extraction steps were performed according to standard phenol/chloroform methods (Sambrook & Russell, 2001). Each set of DNA extractions included a negative control without insect tissue. The DNA pellet was washed, air dried and resuspended in 50 µl of sterilized double distilled H_2O. DNA concentration and purity was assessed by spectrophotometry using the SPECTROstar Nano and the MARS Data Analysis Software (BMG Labtech, Germany).

The ITS2 rDNA fragment was amplified using the forward (ITS2-LEcEn-F) (5′-TGA ACA TCG ACA TTT TGA AC-3′) and the reverse (ITS2-LEcEn-R) (5′-TTC TTT TCC TCC CCT CAT TAA TAT GCT TAA-3′) primers designed with the aid of the Primer Designing Tool available at the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer pair designation was based on homology analysis of relevant rDNA sequences of closely related fly species available online on GenBank. Finally, we chose *Drosophila melanogaster* rDNA sequence (GenBank accession no. M21017) as candidate template. The forward primer sequence is identical to nucleotides 2,805–2,824 of the template. The reverse primer sequence is complementary to template nucleotides 3,328–3,357, with a modification in nucleotide 3,342 (C for T, underlined in the reverse primer sequence).

Amplifications were carried out in a 25 µl reaction volume containing 1× reaction buffer (final concentration 20 mM Tris–HCl, 50 mM KCl, 5 mM MgCl2, pH 8.4), 200 µM dNTP, 2.5 units Taq polymerase

Fig. 1 Map of part of southern South America showing the localities where specimens of *Philornis* spp. were collected for this study. 1 Esperanza city (Santa Fe Province, Argentina), 2 Salto city (Salto Department, Uruguay), 3 Santa Fe city (Santa Fe Province, Argentina), 4 La Paz Department (Mendoza Province, Argentina), 5 Escobar city (Buenos Aires Province, Argentina), 6 Magdalena city (Buenos Aires Province, Argentina), 7 Parque Nacional Chaco (Chaco Province, Argentina). Specimens identified as *P. seguyi* with an ITS2 sequence different from those for *P. torquans* are named "*Philornis* sp. Magdalena". The interrogation sign indicates Misiones Province (Argentina) where *P. seguyi* was described by Garcia (1952) (type-locality unknown). *Key to symbols*: circle, *P. torquans*; square, *P. downsi*; triangle, *P. seguyi*; diamond, unidentified *Philornis* larvae; star, "*Philornis* sp. Magdalena"

(Invitrogen, Brazil), 0.4 pM of each primer, and 100 ng of genomic DNA. PCR was initiated by denaturation at 95 °C for 5 min, followed by 40 cycles of: 45 s at 94 °C for template denaturation, 45 s at 55 °C for primer annealing, and 1.5 min at 72 °C for primer extension. The reaction was terminated by a 5 min elongation cycle at 72 °C. Each PCR run included a negative control with insect-free DNA extraction. To test that no organisms from the gut or mouthparts would contaminate the fly sequences, a set of DNA extractions was performed from fly legs and from specimens in which the abdomen was excised. No differences in the products were observed when compared to DNA extractions from complete specimens (data not shown). All samples were amplified in quadruplicate and a single band of each reaction was visualized in 1.5% agarose gels stained with GelRed™ (Biotium, USA) and photographed under UV light. The size of PCR products was compared with the molecular marker 100 bp DNA Ladder (Genbiotech, Argentina). PCR products were column purified and sequenced directly in both directions using the amplifying primers. Sequencing was conducted under BigDye™ terminator cycling conditions and reacted products were run using an Applied Biosystems 3730xl DNA Analyzer.

The ITS2 sequences were aligned using the ClustalX 2.1 sequence alignment software (Larkin et al., 2007) with ClustalW multiple alignment option with default parameters and without manual optimisation. Phylogenetic analysis was performed with the Molecular Evolutionary Genetics Analysis (MEGA v. 5.02) programme (Tamura et al., 2011) and evolutionary distances were inferred by using the maximum likelihood (ML) method. The ML tree was generated with the Tamura 3-parameter model by using uniform rates. The best fitting substitution model was determined with the Bayesian Information Criterion using the ML model test implemented in MEGA 5. Support for the topology was tested by bootstrapping over 1,000 replications. The ITS2 sequence for *Muscina stabulans* (GenBank accession no. EF061812) was used as an outgroup. The novel sequences were deposited on GenBank under the following accession numbers: *P. torquans* (KC485555-KC485556, KC485560-KC485576) and "*Philornis* sp. Magdalena" (KC485557-KC485559).

Results

Morphological examination of adult *Philornis* spp.

All adult specimens of *P. seguyi* and *P. torquans* showed indistinguishable morphology with respect to yellow antennae and palpi; yellow haired cheeks and brown mesonotum with grey polinosity. The mesonotum had four brown vittae in both *P. seguyi* and *P. torquans*. Measurements of frons width in *P. torquans* were 0.075 ± 0.016 mm in males and 0.187 ± 0.01 mm in females. For *P. seguyi*, these measurements were 0.082 ± 0.006 mm in males and 0.135 ± 0.009 mm in females. The number of setae on the anterodorsal surface of the hind tibia was four in 64 and 55% specimens of *P. torquans* and *P. seguyi* respectively. Among the specimens of *P. seguyi* from Magdalena, 50% had four setae on the anterodorsal surface of the hind tibia. With respect to the number of setae on the anteroventral surface of the hind tibia, 91% of the flies examined, including all specimens of *P. seguyi* collected from Magdalena, possessed three

setae. Moreover, 78% of the male specimens of *P. torquans* had a bowed hind tibia whereas this character was not observed in all male specimens identified as *P. seguyi*. There were no significant differences between the two species in the morphology of male and female genitalia, the cephalopharyngeal skeleton and the posterior spiracle.

Molecular characterisation

According to agarose gel electrophoresis estimations, ITS2 PCR products were approximately 550 bp in length (Fig. 2). The ITS2 PCR products for *P. downsi* were slightly larger than those obtained from the unidentified larvae of *Philornis*, and from the specimens of *P. torquans* and *P. seguyi*.

The sequences obtained from the PCR products ranged in length from 480 bp (*P. seguyi*) to 497 bp (*P. downsi*). In all cases the boundaries of the ITS2 were determined by comparison with *Drosophila melanogaster* rDNA sequence (GenBank accession no. M21017) and 5.8, 2 and 28 S sequences were removed from the analysis. The ITS2 sequences for the specimens of both *P. torquans* and *P. seguyi* were 392–394 nucleotides in length, those for both unidentified larvae of *Philornis* analysed were 394 nucleotides in length whereas those for the three individuals of *P. downsi* were 436 nucleotides in length. The 5.8S, 2S and 28S fragments sequenced were identical in all individuals studied (data not shown). No DNA contamination was detected in the insect-free extractions.

The ITS2 sequences from the specimens of *P. torquans* from Argentina and Uruguay showed 99.5% similarity. The slight difference observed was due to a variation in the number of AC dinucleotide repeats located between nucleotides 93 and 104 of the ITS2 sequence (Fig. 3, dashed rectangle). The alignment of the ITS2 sequences for *P. torquans* (Esperanza) and *P. seguyi* (Santa Fe, La Paz and Magdalena, see Fig. 1) showed mixed results. The ITS2 sequences for *P. seguyi* (specimens from Magdalena) and *P. torquans* showed 94.1% similarity. On the other hand, the sequences for *P. seguyi* based on specimens from Santa Fe and La Paz exhibited 99.5% similarity with those for *P. torquans*. Moreover, two sequences for *P. seguyi* based on specimens from La Paz showed variation in the number of AC dinucleotide repeats identical with that observed in the sequences from specimens of *P. torquans* from Esperanza and Salto (Fig. 3, dashed rectangle). In the light of these results, hereafter all specimens of *Philornis* from Magdalena are referred to as "*Philornis* sp. Magdalena".

The ITS2 sequences of both unidentified larvae of *Philornis* sp. from Escobar city showed 99.5% similarity with *P. torquans* and 94.1% with "*Philornis* sp. Magdalena". Analysis of ITS2 sequence from *P. downsi* showed 65.3% of similarity with *P. torquans* ITS2 sequence and 68.6% with the ITS2 sequences of "*Philornis* sp. Magdalena".

The phylogenetic tree inferred from maximum likelihood analysis of the ITS2 sequences for *Philornis* spp. examined by us is shown in Fig. 4. The analysis showed that larval *Philornis* sp., and adult specimens

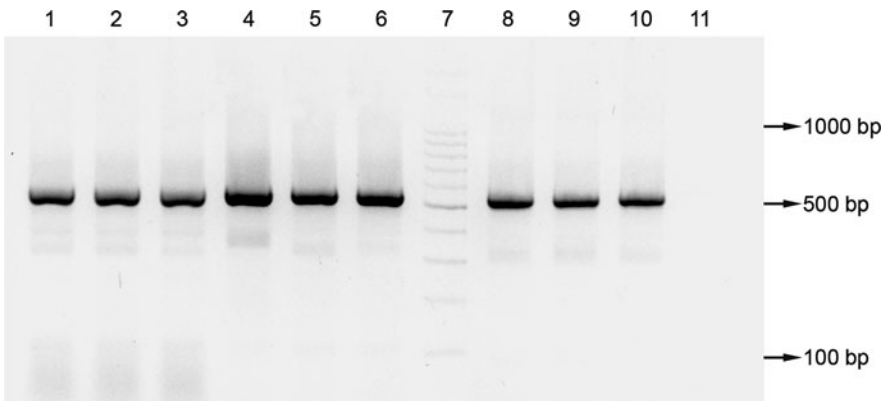

Fig. 2 Inverted image of a 1.5% agarose gel showing ITS2 PCR products from three specimens of each species studied. *Lanes 1–3 P. torquans* (ARGesp), *Lanes 4–6 P. downsi* (ARGcha), *Lane 7* 100 bp DNA Ladder, *Lanes 8–10 P. seguyi* (ARGstf), *Lane 11* insect-free DNA extraction control

(b) Philomis seguyi 17-ARGmgd
(b) Philomis seguyi 22-ARGmgd
(b) Philomis seguyi 24-ARGmgd
(a) Philomis seguyi 1-ARGstf
(a) Philomis seguyi 3-ARGstf
(a) Philomis seguyi 4-ARGstf
(a) Philomis seguyi F3-ARGstf
(a) Philomis seguyi F4-ARGstf
(a) Philomis seguyi C22-ARGstf
(a) Philomis seguyi 10-ARGlpz
(a) Philomis seguyi 11-ARGlpz
(a) Philomis seguyi 12-ARGlpz
Philomis torquans 1-ARGesp
Philomis torquans 3-ARGesp
Philomis torquans 4-ARGesp
Philomis torquans 20-ARGesp
Philomis torquans 21-ARGesp
Philomis torquans 2-URUsal
Philomis torquans 13-URUsal
Philomis torquans 16-URUsal
Philomis larvae 1-ARGesc
Philomis larvae 2-ARGesc
Philomis downsi 1-ARGcha
Philomis downsi 2-ARGcha
Philomis downsi 3-ARGcha

◀**Fig. 3** Multiple alignment of ITS2 sequences from larval *Philornis* sp., and adult specimens of *P. torquans*, *P. seguyi* and *P. downsi*. Asterisks represent identical nucleotides in all sequences and dashes represent gaps introduced to maintain alignment. The site with variation in the number of AC repeats is indicated by a dashed rectangle. Differences between ITS2 sequences for *P. torquans* and *P. seguyi* from Magdalena are highlighted in grey. Sequences marked with (a) are from specimens identified as *Philornis seguyi* (Santa Fe and La Paz) with ITS2 sequences identical to those for *P. torquans*. Sequences marked with (b) are from specimens identified as *Philornis seguyi* (Magdalena) with ITS2 sequences different from *P. torquans*; these sequences are named "*Philornis* sp. Magdalena"

of *P. torquans* and *P. seguyi* form the same cluster, with all specimens of "*Philornis* sp. Magdalena" emerging as a new group from that cluster (Fig. 4). Nevertheless, this branch is supported by a low bootstrap value (62%). Specimens of *P. downsi* were placed in a different cluster.

Discussion

To our knowledge, this is the first study that uses a molecular approach to shed light on the systematics of the species of *Philornis*, representing a new tool to elucidate identifications within the genus. The results also represent the first record of *P. torquans* from Mendoza Province (Argentina) and Uruguay.

Based on the morphological examination of all samples, on a review of species descriptions, and on the key to identification of adults (Couri, 1999; Couri

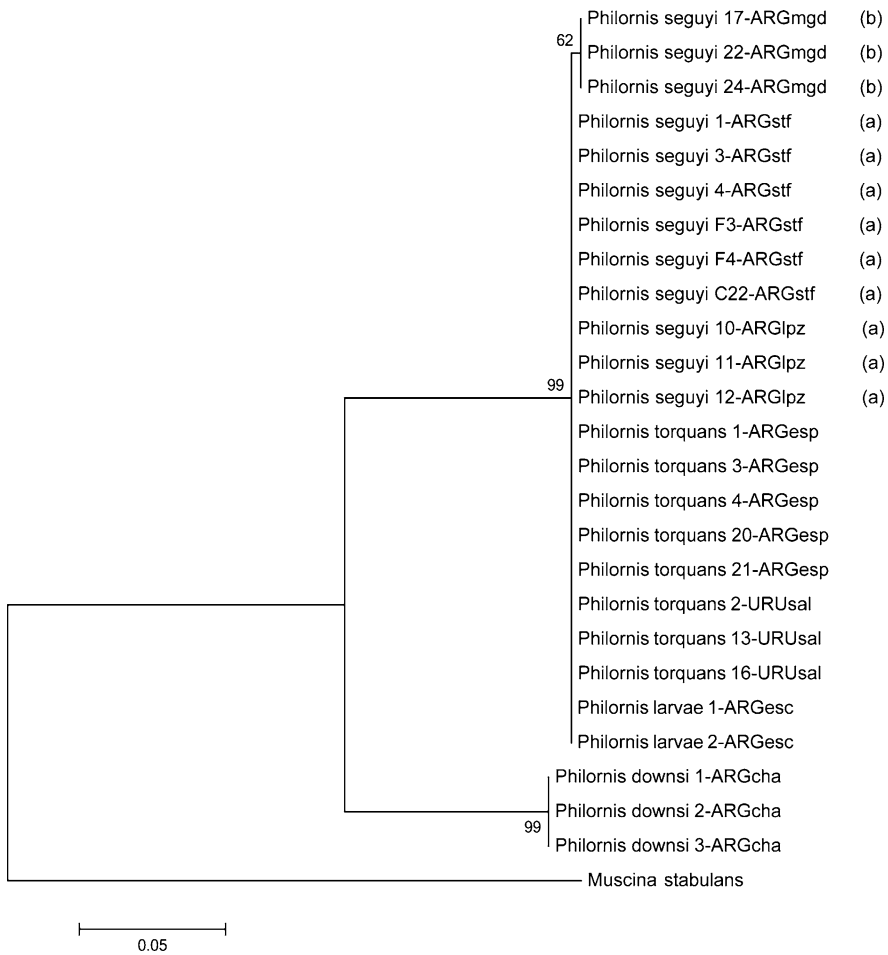

Fig. 4 Phylogenetic analysis of ITS2 sequences from larval *Philornis* sp., and adult specimens of *P. torquans*, *P. seguyi* and *P. downsi*. Bootstrap consensus tree was inferred by maximum likelihood method. Bootstrap values are based on 1,000 replicates and expressed as percentages. Sequences marked with (a) are from specimens identified as *Philornis seguyi* (Santa Fe and La Paz) with ITS2 sequences identical to those for *P. torquans*. Sequences marked with (b) are from specimens identified as *Philornis seguyi* (Magdalena) with ITS2 sequences different from *P. torquans*; these sequences are named "*Philornis* sp. Magdalena"

et al., 2009), it becomes evident that the analysis of external morphological characters presents some weaknesses when used to distinguish *P. seguyi* from *P. torquans*. In this study, specimens identified as *P. seguyi* from Buenos Aires Province showed a distinctive ITS2 rDNA sequences thus confirming that they belong to a species different from *P. torquans.* On the other hand, all specimens from Santa Fe and Mendoza which were morphologically identified as *P. seguyi* showed no difference in their ITS2 sequences when compared with those identified as *P. torquans* from Santa Fe or Uruguay. These results reveal that the specimens of *P. seguyi* from Santa Fe and Mendoza provinces have been misidentified and in fact represent *P. torquans*.

Philornis seguyi was described by Garcia (1952) from a single specimen which is currently lost (Couri et al., 2009). Additionally, the type-locality for this species is erroneously indicated as "Misiones, Argentina" which is an Argentinean Province and not a locality. The latter issue complicates the task of acquiring a neotype for *P. seguyi* and further progress in the morphological and genetic characterisation of this species. Our results strongly suggest that *P. seguyi* may have been misidentified for many years in Argentina. Historically, the presence of *P. seguyi* has been reported in several Argentinean provinces, namely Santa Fe (Quiroga & Reboreda, 2012), Buenos Aires (Couri et al., 2005; Segura & Reboreda, 2011), Cordoba (Nores, 1995) and Misiones (Garcia, 1952). After its original description by Garcia (1952) *P. seguyi* has been reported by Nores (1995), Couri et al. (2005), Rabuffetti & Reboreda (2007), Turienzo & Di Iorio (2007), Segura & Reboreda (2011), and Quiroga & Reboreda (2012, 2013). However, the molecular evidence presented herein indicates that new studies are required in order to elucidate whether the previous records of *P. seguyi* represent misclassified *P. torquans*, or other species. Moreover, the finding of specimens from Magdalena (Buenos Aires Province) identified morphologically as *P. seguyi* with ITS2 sequences different from those of *P. torquans* do not necessarily confirm that they indeed belong to *P. seguyi*, nor that *P. seguyi* is a valid species. Because the ecological context present in Magdalena differs greatly from that of Misiones Province (the type-locality of *P. seguyi*), it becomes essential that material from the latter locality with morphology matching that of *P. seguyi* is subjected to molecular analysis to determine whether this species is valid or not.

Regarding intraspecific genetic diversity, only a slight intraspecific variation in ITS2 sequences was observed in a few specimens of *P. torquans* as a result of a difference in the number of AC dinucleotide repeats. On the other hand, neither "*Philornis* sp. Magdalena" nor the specimens of *P. downsi* showed intraspecific sequence variation; however, only few specimens were analysed. Finally, on the basis of the ITS2 sequences, both larval isolates of *Philornis* from Escobar (Buenos Aires Province) could be identified as *P. torquans*. Altogether, our results show that there is a lack of correspondence between the differences in the morphological characters analysed and the ITS2 sequence among specimens identified as *P. torquans* and *P. seguyi*. Molecular studies like the one carried here would shed light on the yet unclear taxonomy of the genus *Philornis*. Furthermore, the development of *Philornis* species-specific PCR primers taking advantage of the differences in the ITS2 region will allow a quick and inexpensive identification method, even of *Philornis* larvae and pupae.

Acknowledgements We would especially like to thank Leandro Raúl Antoniazzi, Guido Loza and Dante Di Nucci for their assistance in fieldwork. Dr. Hugo H. Ortega kindly made available the facilities and equipment of his Molecular Biology Laboratory to conduct part of the molecular work. All procedures conducted in this study comply with the current National and Provincial laws, and were approved by the Bioethics Committee of Universidad Nacional del Litoral. This work was funded by the Argentine Council for Research and Technology (Grant No. PIP–1122010010026) and by the Morris Animal Foundation (Grant No. D08ZO-304).

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