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A new species of *Hedruris* (Nematoda: Hedruridae) from freshwater turtles, its life cycle and biogeographic distribution of the genus

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Research Paper

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Abstract

We describe *Hedruris dratini* n. sp. (Nematoda, Hedruridae) from *Hydromedusa tectifera* and *Phrynops hilarii* in Argentina based on morphological and molecular characters. Also, we provide information about its life cycle. The new species differs from other species of the genus by possessing the excretory pore, nerve ring and deirids at equal distance from the anterior end. Additionally, *H. dratini* n. sp. has mammilated eggs and males possess nine pairs of caudal papillae. The subadults and adults of *H. dratini* n. sp and *H. orestiae* were characterized by sequencing the small subunit ribosomal DNA (18S). We present for the first time a life cycle of a species of *Hedruris* that includes an amphipod as intermediate host and a reptile as definitive host. Furthermore, we analysed the host and geographic distribution of all *Hedruris* species. Although the genus has a cosmopolitan distribution and parasitizes a great host diversity, the majority of species have a Gondwanian distribution, with amphibians being the preferred hosts.

Introduction

The species of the genus *Hedruris* Nitzsch, 1821 are parasites of the digestive tract of lampreys, fish, amphibians and reptiles, commonly found in their stomach and duodenum. Females attach to the epithelial wall using a characteristic eversible hook situated in their caudal end, while males are found either coiled around the female ones or free in the stomach lumen (Baker, 1982, 1986; Blair, 1984). The genus is widely distributed and comprises 28 species, four of which are considered doubtful, that is, *Hedruris iheringi* Pereira & Vaz, 1933, *H. hylae* Johnston & Mawson, 1941, *H. scabra* Freitas & Lent, 1941 and *H. marinus* Kurochkin & Korotaeva, 1974 (Baker, 1982, 1986; Ramadan *et al.*, 2014; Jones & Resasco, 2016; Rossin & Timi, 2016). In the Neotropical region, nine species of *Hedruris* were recorded: *H. siredonis* Baird, 1858, *H. orestiae* Moniez, 1889, *H. mucronifer* Schuurmans Stekhoven, 1951, *H. basilichtensis* Mateo, 1971, *H. juninensis* Bendezú, 1976, *H. moniezi* Ibañez & Córdova, 1976, *H. heyeri* Bursey & Goldberg, 2007, *H. suttonae* Brugni & Viozzi, 2010 and *H. bifida* Rossin & Timi, 2016. Among them, only *H. mucronifer*, *H. suttonae*, *H. bifida* and *H. orestiae* occur in Argentina (Bursey & Goldberg, 2000; Palumbo *et al.*, 2016; Rossin & Timi, 2016; Serrano-Martinez *et al.*, 2017).

Only two valid species of *Hedruris* were reported in freshwater turtles: *H. pendula* (Leidy, 1851), which parasitizes the painted turtle *Chrysemys picta* (Schneider, 1783), the spotted turtle *Clemmys guttata* (Schneider, 1792) and the Blanding's turtle *Emys blandingii* (Holbrook, 1838) in Canada, United States, France and Germany (in a zoo), and *H. orestiae*, which parasitizes the Argentine snake-necked turtle *Hydromedusa tectifera* Cope, 1870 in Buenos Aires, Argentina (Baker, 1982; Palumbo *et al.*, 2016).

Only two species of the genus were molecularly characterized: (i) Luque *et al.* (2010) sequenced a partial region of the 18S and the third domain of 28S (D3) rRNA genes from 16 isolates of *Hedruris spinigera* Baylis, 1931 from *Retropinna retropinna* Richardson, 1848 and *Paracorophium excavatum* (Thomson, 1884); (ii) Choudhury & Nadler (2018) sequenced a partial region of the18S gene from one isolate of *Hedruris* sp. from salamander *Taricha granulosa* (Skilton, 1849). Thus, little molecular data are accessible at GenBank to make comparative analyses.

Information is available on the life cycles of four species of the genus *Hedruris*: *H. androphora* Nitzsch, 1821 and *H. ijimai* Morishita, 1926 require isopods of the genus *Asellus* Geoffroy, 1762 as intermediate host and amphibians as definitive host (Petter, 1971; Hasegawa & Otsuru, 1979), whereas *H. spinigera* and *H. suttonae* need amphipods as

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intermediate hosts (*Paracorophium* Stebbing, 1899 and *Hyalella* Smith, 1874, respectively) and fishes as definitive hosts (Petter, 1971; Hasegawa & Otsuru, 1979; Brugni & Viozzi, 2010; Luque *et al.*, 2010).

In this work, we describe a new species of *Hedruris* parasitizing two species of freshwater turtles, *Hyd. tectifera* and the Hilaire's toad-headed turtle *Phrynops hilarii* (Duméril & Bibron, 1835), in streams of Buenos Aires province, Argentina, based on morphological and molecular data. In addition, we provide information about its life cycle based on the larval stages recovered from its intermediate host *Hyalella bonariensis* Freitas dos Santos, Araujo & Bond-Buckup, 2008. Moreover, we update the host and geographic distributions of all species in the genus *Hedruris*.

Material and methods

Sampling

Between December 2016 and November 2017, stomach contents and faecal matter were obtained from 47 *Hyd. tectifera* and two *P. hilarii* in the stream Rodríguez (SR) (34°53′02″S, 58° 02′30″W), 16 *Hyd. tectifera* in the stream Carnaval (SC) (34° 52′20″S, 58°05′22″W) and 14 *Hyd. tectifera* in the stream El Gato (SG) (34°53′26″S, 57°59′40″W) in Buenos Aires province, Argentina. The geodetic datum of the geographic coordinates is WGS84. Stomach washes were collected according to the methods of Legler (1977) and then preserved in 70% ethanol. Faecal samples were preserved in 70% ethanol. All samples were analysed using a stereoscopic microscope (Olympus SZ61, Tokyo, Japan); the nematodes and prey items were quantified and preserved in 70% ethanol. In addition, a single *Hyd. tectifera* specimen found dead on the road to the stream Rodríguez in October 2017 was necropsied. It was fixed in 10% formaldehyde for further analysis.

In order to find larval stages in the intermediate hosts, we examined the crustaceans found in the stomach contents of the turtles. Moreover, 100 amphipods were collected in the stream Rodriguez by a plankton net.

Morphological identification

For morphological studies under a compound microscope (Olympus BX51, Tokyo, Japan), nematodes were temporarily mounted and cleared in Amman's Lactophenol. Additionally, photographs were taken with a Q-Imaging Go-3 digital camera. Drawings were made with the aid of a camera lucida. Some specimens were dehydrated, critical point dried, gold coated and observed and photographed using a scanning electron microscope (Jeol/SET 100, Tokyo, Japan). Nematodes were identified according to Yamaguti (1961), Ibañez & Córdova (1976), Baker (1986) and Moravec & Vargas-Vázquez (1998). Measurements are given in micrometres unless otherwise indicated. The number (*n*), prevalence (P) and mean intensity (MI) of nematodes were calculated following Bush *et al.* (1997) and using the Quantitative Parasitology Program (QP 3.0, Rozsa *et al.*, 2000). Voucher specimens were deposited in the Helminthological Collection of the Museo de La Plata, La Plata, Buenos Aires province, Argentina.

The crustaceans were cleared using Amman's Lactophenol and examined for endoparasites under a stereoscopic microscope.

Molecular and polymerase chain reaction (PCR) methods

A total of 14 individual nematodes were isolated in Eppendorf® tubes and frozen at −20°C for subsequent DNA extraction and genotyping: nine adult specimens from the stomach of *Hyd. tectifera* (five males and four females) and five subadults from *Hy. bonariensis*. DNA was extracted from the isolates using 200 µl of 5% Chelex solution (Bio-Rad Laboratories, CA, USA) containing 0.2 mg/ml Proteinase K (Roche), incubated at 56°C overnight, followed by 10 min at 95°C. Nuclear 18S rDNA amplification was done using primers Nem_18S_F and Nem_18S_R (Floyd *et al.*, 2005), which span approximately 900 bp of the gene Each 50 µl PCR contained 25 µl of GoTaq Green Master Mix (Promega, Madison, WI, USA), 2.5 µl of each primer, 17 µl of water and 3 µl of extracted DNA. PCR amplifications for 18S were performed using an Eppendorf Mastercycler ep gradient S (Thermal Cycler 96 WELL, Hamburg, Germany), consisting of 94°C for 15 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 70 s, with a final extension of 72°C for 240 s. PCR products were further purified and sequenced (Macrogen, South Korea). Sequences were edited and aligned using Chromas version 2.6.6® and Gap version 4.11.2® (Bonfield *et al.*, 1995) then compared to the NCBI database using BLAST version 2.2.26 (Altschul *et al.*, 1997) to identify sequences with high similarity to DNA sequences obtained.

Phylogenetic analyses

The newly obtained 18S sequences were aligned with other sequences of *Hedruris* available in the GenBank using CLUSTAL W program (Larkin *et al.*, 2007). The phylogenetic tree was reconstructed using the Maximum Likelihood method and the genetic distance was estimated using Kimura's (1980) two-parameter (K2-P) model implemented in the Mega 7.0.26 program (Kumar *et al.*, 2016). A sequence of one Spiruridae species (GenBank accession number JQ771746) was included as outgroup. Bootstrap analyses were conducted using 500 replicates. The genetic differences in datasets were also calculated with Mega program, using uncorrected *p*-distances.

Host and geographic distribution

To estimate the host and geographic distribution of the genus *Hedruris*, an exhaustive analysis was made of the available bibliography up to 2018. Publications in indexed journals and records in renowned books were taken into account.

Results

A total of 2573 nematodes were obtained from the visceral dissection, stomach washes and faecal samples of the turtles examined, whereas 24 nematodes (one fourth-stage larva and 23 subadults) were recovered from the *Hy. bonariensis* specimens collected from the stream Rodriguez. Every time amphipods were found in the turtle's stomach contents, at least one of them was parasitized. Both adult and larval nematodes found belonged to the genus *Hedruris*. Only ten adult and one subadult specimens from eight *Hyd. tectifera* and one subadult found in *Hy. bonariensis* from the stream Rodriguez were identified as *H. orestiae*. The remainder nematodes belonged to a new species of the genus *Hedruris*, which is described in the following.

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Fig. 1. Male of *Hedruris dratini* n. sp. encircling female. Scale bar: 500 µm.

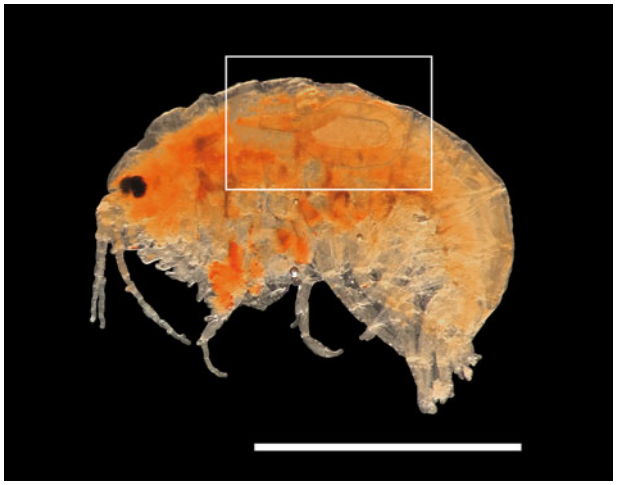

Fig. 2. *Hyalella bonariensis* with subadult of *Hedruris dratini* n. sp. (white square). Scale bar: 1 mm.

Systematics

Hedruridae Railliet, 1916
Hedrurinae Chitwood & Wehr, 1934
Hedruris Nitzsch, 1821
***Hedruris dratini* n. sp.**

Taxonomic summary

Type host. Hydromedusa tectifera Cope, 1870 (Pleurodira, Chelidae).

Other hosts. Phrynops hilarii (Duméril & Bibron, 1835) (Pleurodira, Chelidae); *Hy. bonariensis* Freitas dos Santos, Araujo & Bond-Buckup, 2008 (Amphipoda).

Type locality. Stream Rodríguez (34°53′02″S, 58°02′30″W; datum: WGS84), Buenos Aires province, Argentina.

Other localities. Stream El Gato and stream Carnaval, Buenos Aires province, Argentina.

Type material. Holotype: MLP-He-7510 (male). Paratypes: MLP-He-7511 (two males and two females).

Site of infection. Adults in the stomach of turtles (fig. 1), larvae in the haemocoel of amphipods (fig. 2).

Prevalence (P) and mean intensity (MI) in definitive hosts. Hydromedusa tectifera. SR: n = 2286, P = 97.8%, MI = 51 (2–198); SG: n = 2, P = 14%, MI = 1; SC: n = 136, P = 43%, MI = 19.4 (1–119). Phrynops hilarii. SR: n = 3, P = 100%, MI = 1.

Prevalence (P) and mean intensity (MI) in the intermediate host. Hyalella bonariensis. SR: n = 100, P = 23%, MI = 1.

Etymology. The specific name is an arbitrary combination of two words (dra = dragon; tini = tiny).

General description (based on 40 specimens) (figs 3 and 4). Body cuticle thick, transversally striated. Cephalic end with two large lateral pseudolabia, each bearing a pair of small apical digitiform papillae, a pair of lateral sessile papillae and an amphid. Base of each pseudolabium supported by posteriorly directed cuticular ridge. Dorsal and ventral interlabium between pseudolabia, each with anteriorly directed lobe, curved over surface of apical region, and two bifurcated lateral cuticular structures; cuticular ridge posteriorly directed between bifurcations. Base of each interlabium supported by a large, posteriorly directed ridge. Buccal cavity thin-walled, oesophagus not clearly divided into muscular and glandular portions. Deirids simple, situated at level of excretory pore and just anterior to nerve ring. Female with posterior sclerotized hook for attachment to host; male generally found encircling female.

Male (based on 20 specimens measured) (figs 3b and 4c). Body length 5.97 (3.64–9.24) mm, maximum body width 203 (138–275). Oesophagus 1.07 (0.8–1.27) mm long. Deirids and excretory pore at same level, 200 (148–245) from anterior end. Nerve ring 220 (168–265) from anterior extremity. Posterior end of body with 1–3 coils. Nine pairs of postcloacal papillae: eight pairs subventral and one pair lateral, situated between second last and last pair of subventral papillae. A pair of tiny ventro-lateral phasmids, just posterior to last pair of subventral papillae. Area rugosa with ten ventral longitudinal ridges with scale-like knobs. Spicules 182 (169–233) long, markedly slender, fused in their distal two-thirds, with membranous expansions. Gubernaculum lacking.

Female (based on 20 gravid specimens measured) (figs 3c–e and 4d). Body length 9.1 (7.09–10.94) mm, maximum body width 424 (215–571). Oesophagus 1.7 (1.3–1.8) mm long. Deirids and excretory pore at same level, 270 (252–285) from anterior end. Nerve ring 295 (275–340) from anterior end. Vulva 716 (465–1127) anterior to anus. Tail bulbous, curved dorsally, with eversible prehensile structure (holdfast) armed with sclerotized hook. Cuticular spines in posterior part of body, distributed in two areas; one with scale-like spines near region of anus, the other with sharply pointed spines on the dorso-lateral surface of holdfast. Didelphic, prodelphic. Egg-filled uteri occupying body cavity completely when gravid. Mammilated eggs 36 (27–41) long and 19 (13–24) wide, operculated at both poles, containing fully developed larva.

Fourth-stage larva (based on one specimen found in *Hy. bonariensis*) (fig. 5). Cephalic end poorly developed, with interlabia and pseudolabia, but with little development of posterior bridges at base of lips. Digestive tract simple with an anal plug. Cuticle of previous moult observed in posterior region of body. Male larvae with well-developed testicle and a pouch in final part of intestine where primordial spicules appear.

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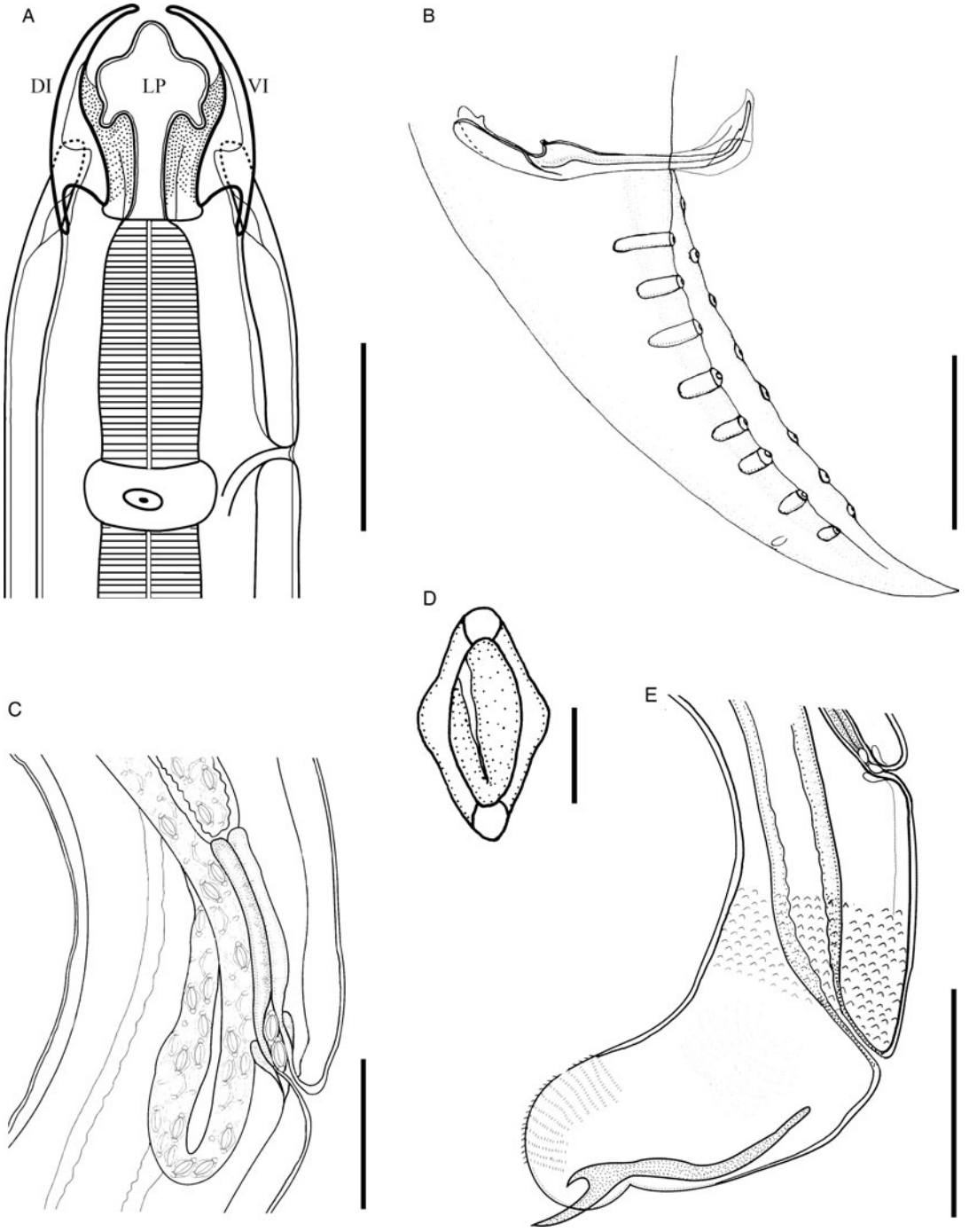

Fig. 3. Line drawings of *Hedruris dratini* n. sp. (A) Anterior end of female, lateral view; (B) caudal end of male, sublateral view; (C) vulva of female, lateral view; (D) egg; (E) posterior end of female, lateral view. Scale bars: (A, B) 100 µm; (C) 200 µm; (D) 20 µm; (E) 500 µm. Abbreviations: LP, lateral pseudolabium; DI, dorsal interlabium; VI, ventral interlabium.

Subadult (based on 23 specimens found in *Hy. bonariensis*). Morphological characteristics of this stage seem to be identical to the adults previously described, but these differ in size and in the degree of development of their reproductive organs (e.g. smaller size of the testes in males and absence of eggs in females).

Remarks

Hedruris dratini n. sp. can be easily distinguished from all other species of the genus by the position of the deirids in relation to the excretory pore. Both structures are located at the same level in the new species, whereas the excretory pore is located far posterior to the deirids in the rest of the species of the genus. The features commonly used to distinguish species of *Hedruris* are the shape of the eggs and the number and distribution of the caudal papillae in males (Bursey & Goldberg, 2000). Among the 24 valid species described to date, only *H. androphora*, *H. siredonis* and *H. ijimai* have mammilated eggs, as in *H. dratini* n. sp. (Baird, 1858; Petter, 1971; Hasegawa & Otsuru, 1979). However, males of *H. androphora* and *H. ijimai* possess precloacal and

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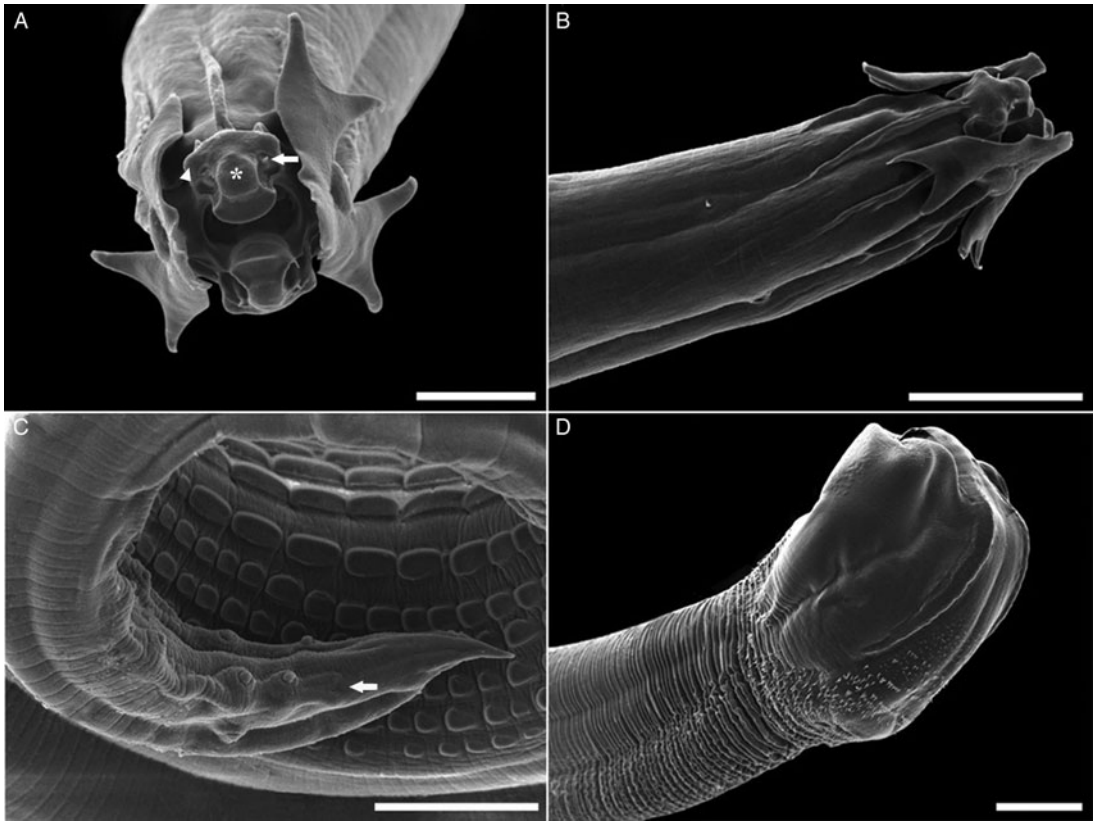

Fig. 4. Scanning electron micrographs of *Hedruris dratini* n. sp. (A) Apical view of male (arrowhead: sessile papillae; arrow: digitiform papillae; asterisk: amphid); (B) anterior end of male, sublateral view; (C) tail of male, ventral view (arrow: phasmid); (D) posterior end of female, subdorsal view. Scale bars: 100 µm.

adcloacal papillae (Petter, 1971; Hasegawa & Otsuru, 1979), which are absent in the new species. *Hedruris siredonis* can also be distinguished from the new species by the distribution and number of caudal papillae in males: 20–2:2:16 (total number of papillae–precloacal:adcloacal:postcloacal + anterior cloacal lip) in *H. siredonis* vs. 18–0:0:18 in *H. dratini* n. sp. (Baird, 1858).

There are five species with the same distribution of caudal papillae in males as *H. dratini* n. sp. (i.e. 18–0:0:18), all of them from the Neotropics: *H. orestiae*, *H. basilichtensis*, *H. moniezi*, *H. suttonae* and *H. bifida* (Bursey & Goldberg, 2000; Rossin & Timi, 2016). However, none of them possess mammilated eggs. Additionally, females of *H. moniezi* are described as opistodelphic, those of *H. suttonae* and *H. bifida* are monodelphic, but *Hedruris dratini* n. sp. females are didelphic, prodelphic (Ibañez & Córdova, 1976; Brugni & Viozzi, 2010; Rossin & Timi, 2016).

Molecular analyses

A total of six sequences (up to 889 bp) were yielded from subadults and adults of *H. dratini* n. sp. (GenBank accession numbers MK928970, MN233055–MN233060). The alignment of these sequences returned 701 bp fragments, which exhibited 99.43% identity to each other, a single polymorphic site and three insertion/deletion events. A sequence of 840 bp was also yielded from a subadult male of *H. orestiae* (GenBank accession number MN263058). By BLAST similarity analysis, the correct 18S gene amplification was confirmed and no identical matches were found. The sequences obtained from *H. dratini* aligned closest to *H. spinigera* from New Zealand smelt (GenBank accession

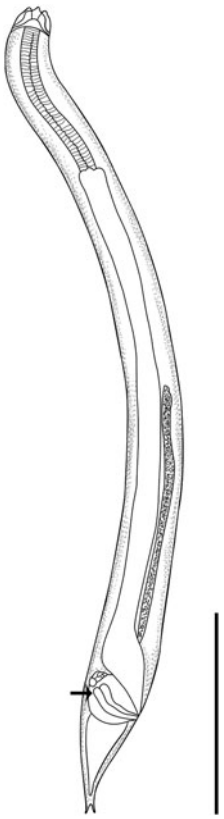

Fig. 5. Line drawing of male fourth-stage larva of *Hedruris dratini* n. sp. with primordial spicules (arrow), lateral view. Scale bar: 500 µm.

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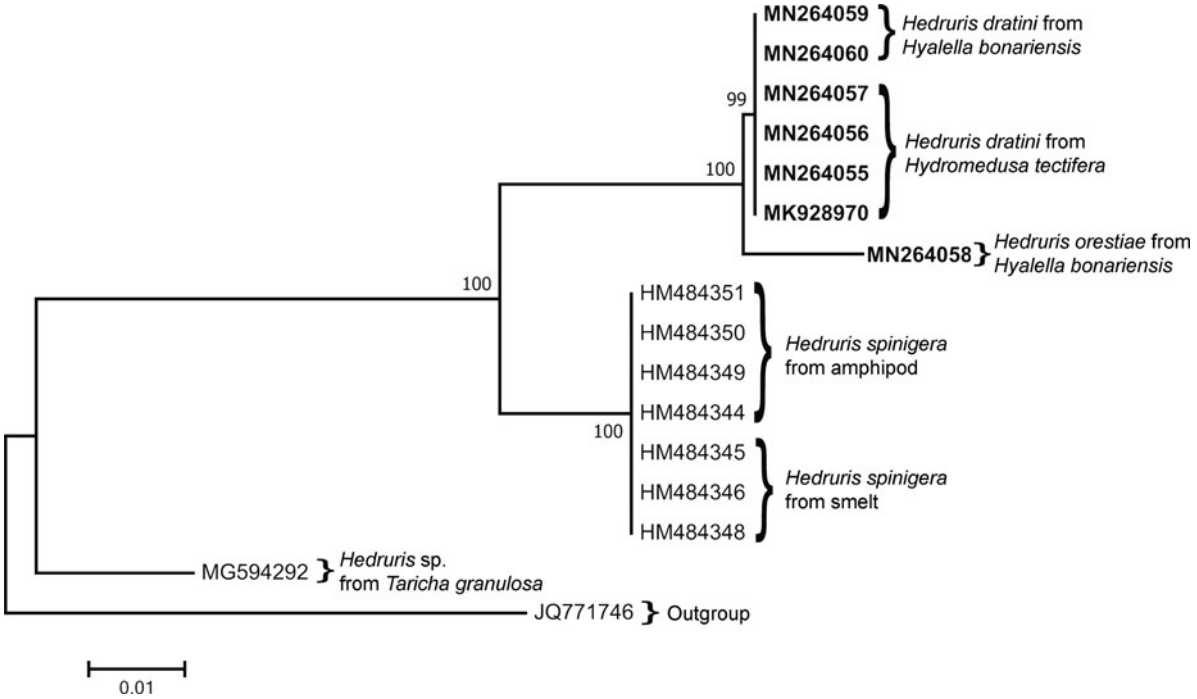

Fig. 6. Phylogenetic tree based on *Hedruris* rDNA sequences newly obtained in this study (bold type) compare with all *Hedruris* sequences available in GenBank using Maximum Likelihood method with a distance matrix calculation with K2P. A Spiruridae species was used as outgroup. The numbers at the nodes represent the percentages of 500 bootstrap replicates. Sequences are identified by GenBank accession numbers, taxa names and hosts.

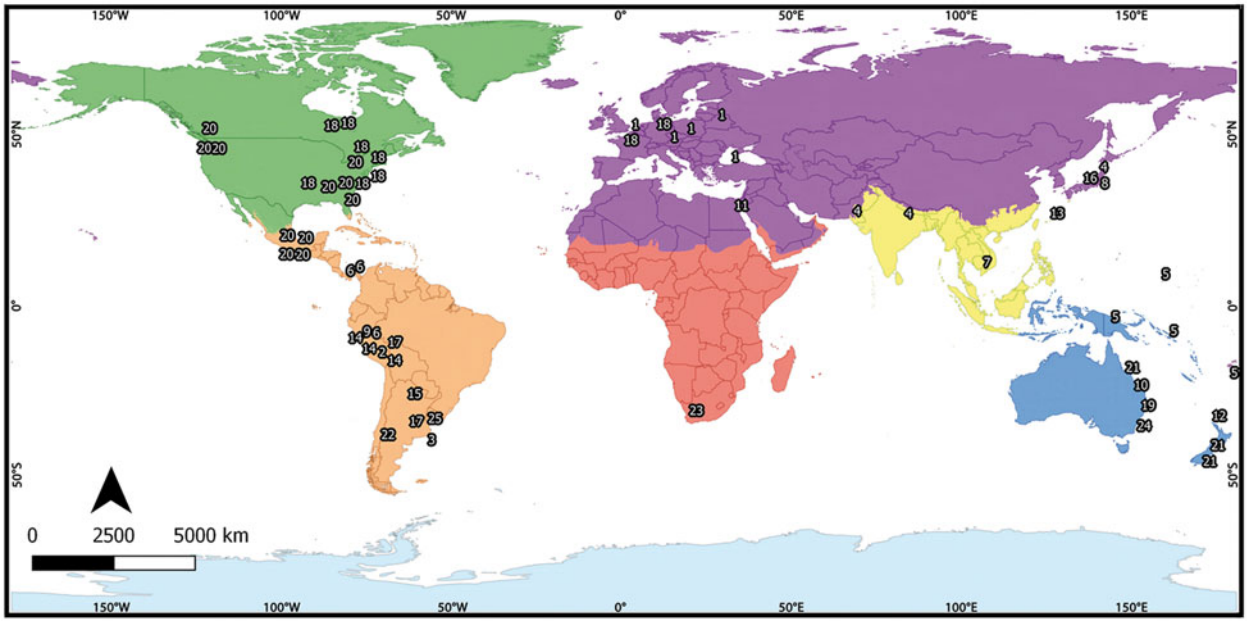

Fig. 7. Map of geographic distribution of *Hedruris* species: (1) *H. androphora*; (2) *H. basilichtensis*; (3) *H. bifida*; (4) *H. bryttosi*; (5) *H. hanleyae*; (6) *H. heyeri*; (7) *H. hipsirhinae*; (8) *H. ijimai*; (9) *H. juninensis*; (10) *H. longispicula*; (11) *H. lutjanenses*; (12) *H. minuta*; (13) *H. miyakoensis*; (14) *H. moniezi*; (15) *H. mucronifer*; (16) *H. neobythitis*; (17) *H. orestiae*; (18) *H. pendula*; (19) H. *saltuarii*; (20) *H. siredonis*; (21) *H. spinigera*; (22) *H. suttonae*; (23) *H. transvaalensis*; (24) *H. wogwogensis*; (25) *H. dratini* n. sp. Colours indicate the biogeographical provinces proposed by Udvardy (1975): green, Nearctic; orange, Neotropics; red, Afrotropics; violet, Palearctic; yellow, Indo-Malaya; blue, Australia; light violet, Oceania.

number HM484346) (95–96% identity), followed by *Hedruris* sp. from American *T. granulosa* (GenBank accession number MG594292) (93–94% identity).

The tree topology showed the isolates from subadults and adults of *H. dratini* n. sp. clustered together with a high bootstrap support of 99%, and very closely with the isolate of *H. orestiae* obtained in our study (100% bootstrap support). The isolates of *H. spinigera* from New Zealand smelt grouped as a sister clade (fig. 6).

Pairwise analyses showed that the genetic difference between *H. dratini* n sp. and *H. orestiae* was 1.3%; between *H. dratini* n sp. and *H. spinigera* ranged from 4 to 4.1%; and between *H. dratini* n. sp. and *Hedruris* sp. from *T. granulosa* ranged from 7 to 7.2%.

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Table 1. Host and geographic distribution of *Hedruris* Nitzsch, 1821 species.

Species	Host	Locality	Reference
H. androphora	*Anguilla anguilla* (fish)	Neusied, Austria	Kritscher (1988)
	Bombina bombina (frog), *Triturus cristatus*, *T. vulgaris* (salamander)	Belarus	Shimalov (2009)
	Bombina bombina, *Bufo calamita* (Anura), *Triturus cristatus*, *T. aplestris* (salamander)	Ukraine	Ryzhikov *et al.* (1980)
	Bombinator igneus, *Bufo calamita* (frog), *Proteus anguinus* (salamander)	Europe	Chandler (1919)
	Pelophylax esculentus, *Rana arvalis* (frog)	Poland	Okulewicz *et al.* (2014)
	Triturus cristatus (salamander)	Lake Abant, Turkey	Schad *et al.* (1960)
	Triturus vulgaris (salamander)	Maroeuil, France	Schneider (1866); Chandler (1919); Petter (1971); Baker (1982)
H. basilichtensis	*Basilichthys bonariensis*, *Orestias albus*, *O. luteus*, *Salmo gairdneri* (fish)	Puno, Peru	Bendezú (1974); Baker (1982); Sarmiento *et al.* (1999)
H. bifida	*Oligosarcus jenynsii* (fish)	Buenos Aires, Argentina	Rossin & Timi (2016)
H. bryttosi	*Arius arius* (fish)	Karachi, Pakistan	Sattar *et al.* (2016)
	Bryttosus kawamebari, *Mogurunda obscura* (fish)	Japan	Freitas & Lent (1941); Baker (1982)
	Mystus sp. (fish)	Nepal	Jha & Bhujel (2012)
***H. dratini* n. sp.**	*Hydromedusa tectifera*, *Phrynops hilarii* (turtle)	Buenos Aires, Argentina	This paper
H. hanleyae	*Emoia atrocostata* (lizard)	Papua, New Guinea	Goldberg *et al.* (2018)
	Emoia physicae (lizard)	Malaysia	Bursey & Goldberg (2016)
	Emoia boettgeri, *E. caerulocauda*, *E. cyanogaster*, *E. impar*, *E. nigra*, *E. nigromarginata*, *E. sanfordi*, *E. trossula*, *Gebyra mutilata*, *G. oceanica*, *Hemidactylus frenatus*, *Lepidodactylus lugubris*, *L. moestus*, *L. paurolepis* (lizard)	Micronesia	Goldberg *et al.* (2005)
	Hemidactylus garnotii (lizard)	Cook Islands	Bursey & Goldberg (2000)
	Nactus multicarinatus, *N. pelagicus* (lizard)	Vanuatu	Goldberg *et al.* (2011)
H. heyeri	*Craugastor flishmanni*, *C. megacephalus* (frog)	Costa Rica	Goldberg & Bursey (2010)
	Craugastor gollmeri, *Eleutherodactylus caryophyllaceus*, *E. cruentus* (frog)	Isla Bonita, Costa Rica	Goldberg & Bursey (2008)
	Lithobates warszewitschii (frog)	Puntarenas, Costa Rica	Bursey & Goldberg (2007)
	Telmatobius jelskii (frog)	Lima, Peru	Serrano-Martinez *et al.* (2017)
H. hipsirhinae	*Hipsirhina bocourti* (snake)	Cochinchina, China	Chatin (1876); Baker (1986)
H. ijimai	*Rana japanica*, *R. nigrimaculata, R. rugosa* (frog)	Chiba, Japan	Freitas & Lent (1941); Baker (1982); Shimazu & Araki (2006)
H. juninensis	*Batrachophrynus brachydactylus*, *B. macrostomus* (frog)	Junín, Peru	Bendezú (1974); Baker (1982); Sarmiento *et al.* (1999)
H. longispicula	*Lygosoma challengeri* (lizard)	Queensland, Australia	Baker (1982)
H. lutjanenses	*Lutjanus synagris* (fish)	Damietta, Egypt	Ramadan *et al.* (2014)
H. minuta	*Leiolopisma smithi* (lizard)	Waitangi, New Zealand	Andrews (1974); Baker (1982)
H. miyakoensis	*Scincella boettgeri* (lizard)	Okinawa, Japan	Hasegawa (1989)
H. moniezi	*Rhinella flavolineatus* (frog)	Cusco, Peru	Sarmiento *et al.* (1999)
	Rhinella spinulosa, *Telmatobius jelskii*, *T. marmoratus*, *T. peruvianus* (frog)	Lima, Peru	Chero *et al.* (2014, 2016)
	Telmatobius sp. (frog)	Puno, Peru	Bendezú (1974); Baker (1982)
H. mucronifer	*Telmatobius schreiteri* (frog)	Tucumán, Argentina	Schuurmans Stekhoven (1952); Baker (1982)
H. neobythitis	*Neobythites macrops* (fish)	Japan	Freitas & Lent (1941); Yamaguti (1961); Baker (1982)

(*Continued*)

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Table 1. (*Continued.*)

Species	Host	Locality	Reference
H. orestiae	*Orestias agassi*, *O. luteus*, *O. mullen*, *Salmo gairdneri*, *Trichomycterus rivulatus* (fish)	Puno, Peru	Ibañez & Córdova (1976); Baker (1982); Sarmiento *et al.* (1999)
	Hydromedusa tectifera (turtle)	Buenos Aires, Argentina	Palumbo *et al.* (2016)
H. pendula	*Clemmys guttata* (turtle)	Massachusetts, USA, and Zoological Garden, Germany	Leidy (1851); Baker (1986)
	Esox niger (fish)	New York, USA	Baker (1982, 1986)
	Clemmys guttata (turtle)	Ontario, Canada	Baker (1986)
	Chrysemys picta (turtle)	Massachusetts, USA and Zoological Garden, France	Baker (1986)
	Emydoidea blandingi (turtle)	Ontario, Canada	Baker (1986)
	Rana catesbeiana (frog)	Canada	Baker (1986)
	Desmognathus brimleyorum (salamander)	Arkansas, USA	McAllister *et al.* (1995)
H. saltuarii	*Saltuarius moritzi* (lizard)	New South Wales, Australia	Jones (2013)
H. siredonis	*Ambystoma cingulatum* (salamander)	Florida, USA	Whiles *et al.* (2004)
	Ambystoma gracile, *Triturus torosus* (salamander)	Oregon, USA	Chandler (1919); Freitas & Lent (1941); Thorson (1956); Baker (1982)
	Ambystoma mexicanum (salamander)	Mexico	Baird (1858)
	Ambystoma ordinarium, *Ambystoma* sp. (salamander)	Michoacán, Mexico	Baker (1986)
	Ambystoma taylori, *A. tigrinum* (salamander)	Puebla, Mexico	Baker (1982, 1986)
	Ambystoma tigrinum (salamander)	DF, Mexico	Baker (1986)
	Desmognathus fuscus (salamander)	Georgia, USA	Baker (1986)
	Rana clamitans (frog)	New Hampshire, USA	Muzzall & Baker (1987)
	Taricha granulosa (salamander)	Oregon, USA and British Columbia, Canada	Baker (1986)
	Triturus viridescens (salamander)	North Carolina, USA	Walton (1930); Baker (1982)
H. spinigera	*Agonostomus forsteri*, *Anguilla aucklandii*, *A. australis*, *A. diefenbachii*, *Galaxias attenuatus*, *G. maculatus*, *Gobiomorphus basalis*, *Oncorhynchus tschawytscha*, *Retropinna retropinna*, *Rhombosolea plebeia*, *R. tapirina*, *Salmo trutta* (fish)	Christchurch, New Zealand	Baylis (1931); Stokell (1936); Webb (1973); Clark (1978); Baker (1982, 1986); Jellyman (1989)
	Aldrichetta forsteri, *Retropinna retropinna*, *Galaxias maculatus*, *Perca fluviatilis*, *Rhombosolea retiaria*, *Salmo trutta* (fish)	South Island, New Zealand	Luque *et al.* (2010)
	Anguilla reinhardtii (fish)	Queensland, Australia	Kennedy (1995)
	Geotria australis (lamprey), *Leptoscopus macropygus*, *Neochanna apoda* (fish)	New Zealand	Blair (1984)
H. suttonae	*Galaxias maculatus*, *G. platei* (fish)	Rio Negro, Argentina	Brugni & Viozzi (2010)
H. transvaalensis	*Breviceps sylvestris* (frog)	Woodbush, South Africa	Baker (1982); Bursey & Goldberg (2000)
H. wogwogensis	*Lampropholis guichenoti* (lizard)	New South Wales, Australia	Jones & Resasco (2016)

Double infection

We occasionally found adults of *H. orestiae* and *H. dratini* n. sp. within the same host specimen. In some cases, *H. orestiae* females and *H. dratini* n. sp. of both sexes were observed together and none of the *H. dratini* n. sp. males were coiled around those females. In other cases, *H. orestiae* males were found together with *H. dratini* n. sp. of both sexes and, similarly, none of those males were coiled around *H. dratini* n. sp. females. In all these cases, *H. orestiae* females were non-gravid and we never found males and females of *H. orestiae* infecting the same individual host.

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Host and geographic distribution

Including the present paper, a total of 123 literature records have been found for *Hedruris* species worldwide (fig. 7, table 1). Both the reported species richness and number of records are greater in the southern than in the northern hemisphere (17 vs. nine species, and 75 vs. 48 records, respectively). Amphibians as definitive hosts show the highest number of records (46, of which 26 in frogs and 20 in salamanders), followed by 41 records in fish, 35 in reptilians (24 in lizards, ten in turtles and one in snakes) and only one record in lampreys.

Discussion

As regards their life cycles, *Hedruris* species require an isopod or amphipod as intermediate host in which all larval stages develop up to the subadult stage. Subsequently, sexual maturity is reached in the definitive host (Anderson, 2000). Petter (1971) found *H. androphora* in the salamander *Triturus vulgaris* (Linneus, 1758) and studied its development in the isopod *Asellus aquaticus* (Linneus, 1758) by allowing them to ingest larvated eggs. First-stage larvae invade the isopod haemocoel close to the cephalic end. Subsequently, development to the subadult stage takes place, but sexual maturity is only reached in the stomach of the definitive host (Anderson, 2000).

In the *Hy. bonariensis* specimens collected from both the stomach contents and the natural aquatic environment, subadults of *H. dratini* n. sp. were found inside the haemocoel or penetrating the amphipod's cuticle towards the stomach lumen of the definitive host. Subadults have all the characteristics of the adults and are ready to leave the intermediate host, invade the stomach of the definitive host and reproduce, as was described by Moravec (1998). Mature and gravid *H. dratini* n. sp. were found both in the stomach and faeces of the examined turtles. Larvated eggs were also present in the faeces. It can, therefore, be deduced that the life cycle of *H. dratini* n. sp. begins when the female releases the eggs in the digestive tract of the turtle, which are later expelled together with the faeces. Once in the aquatic environment, *Hy. bonariensis* ingest the nematodes eggs, which hatch inside the digestive tract. The first-stage larva migrates to the haemocoel and moult repeatedly to reach the subadult stage. When a turtle swallows the infected amphipods, the subadult is released and reaches sexual maturity in the stomach. Eggs are released with the faeces into the environment, repeating the cycle. This is the first report of the life cycle of a species of *Hedruris* parasite of reptilians.

According to Mayr (1942: 247), 'species are groups of natural populations that potentially interbreed, and are reproductively isolated from other similar groups', which is evidenced by these two co-occurring species (*H. dratini* n. sp. and *H. orestiae*), reinforcing the aforementioned morphological distinction between them. We are convinced that the low prevalence of *H. orestiae* found is due to the interspecific competition with *H. dratini* n. sp., which dominates over *H. orestiae* in this population of *Hyd. tectifera*. These results contrast with Palumbo *et al.* (2016), who found only *H. orestiae* in *Hyd. tectifera* in a stream 100 km away from the study area of the present study.

Sequencing the 18S rRNA gene is a valuable tool for the characterization of isolates to the genus or species level (Liu, 2011; Yooyangket *et al.*, 2018). Present molecular results support the classification of the new species into the genus *Hedruris*, corroborating that *H. dratini* n. sp. differs from the other two species submitted in the GenBank (Luque *et al.*, 2010; Choudhury & Nadler, 2018), because the identity is less than 97%. Additionally, present findings confirm that larval nematodes found in amphipods are the same species as those found in turtles. The partial sequences of 18S gene analysed in this study are highly conserved among the *Hedruris* species. Although the genetic distance between *H. dratini* n. sp. and *H. orestiae* is low, the morphological characters distinguish these two closely related species, which share both the intermediate and definitive host. This point deserves further molecular analysis using other genetic markers (e.g. COI, ITS), which tend to be more variable, providing more reliable species-specific identification (Avise, 1994; Palomares-Rius *et al.*, 2017). However, the obtained sequence information can be considered as a useful resource for taxonomic and phylogenetic studies.

Despite a broad distribution worldwide, South America is the region with the greatest number of species described. Even if areas in which few or no studies have been conducted are taken into account, the distribution map of *Hedruris* species illustrates that members of the genus follow a Gondwanian distribution (fig. 7, table 1). According to the biogeographic provinces proposed by Udvardy (1975), *Hedruris* species are mostly distributed in the Neotropics (ten species, 38 reports), followed by Australia (six species, 30 reports), the Palearctic (six species, 20 reports), the Nearctic (two species, 16 reports), Oceania (one species, 15 reports), Indo-Malaya (three species, three reports) and the Afrotropics (one species, one report). This analysis allows us to hypothesize that the speciation and radiation of *Hedruris* occurred first in Gondwana, probably in fish, and that, subsequently, species dispersed to other hosts as well as to the northern hemisphere because of the great plasticity and host adaptability of the genus.

Hedruris dratini n. sp. is the fifth valid species of the genus in Argentina, which suggests a great adaptive success of the genus in this country.

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