

Rapid Communication**Occurrence in Mexico of two European invasive slug species:
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OPEN ACCESS**Abstract**

Knowledge of invasive terrestrial gastropods species in Mexico, and their potential impacts, is practically unknown. We document in this study, the occurrence of the large invasive slug *Arion vulgaris* Moquin-Tandon, 1855 for the first time in Mexico and only the third time in North America. In addition, we also report *Arion (Kobeltia) intermedius* Norman, 1852 from seven localities demonstrating that the species is already well established in Mexico. To identify both species, a Maximum-Likelihood phylogenetic analysis was performed using partial sequences, alone or concatenated, of nicotinamide adenine dinucleotide dehydrogenase 1 (ND1) and cytochrome C oxidase 1 (COI) genes. In addition, we used the morphological characters reported in other studies of these species to confirm their taxonomic identity. The phylogenetic analysis performed with the concatenated ND1 and COI genes, showed that the Mexican sequences of *A. vulgaris* were grouped with the corresponding sequences of this species from Canada and European countries. In addition, attributes such as the shape and position of the ligula were useful to confirm the taxonomic identity of this species. On the other hand, the phylogenetic analysis using ND1 sequences alone clustered Mexican specimens of *A. intermedius* with sequences of this species from Spain and UK. Characters such as the body color pattern, prickles on the dorsum, as well as characters of the reproductive system of *A. (K.) intermedius* corroborated the taxonomic identity of Mexican specimens of this species.

Key words: Arionidae, invasive slugs, molecular identification, North America**Introduction**

The settlement of invasive species in areas outside their natural distribution can cause damage to biodiversity and ecosystems, and also produce negative effects on the human, animal and plant health. The environmental, economic and social impact of invasive slugs in Mexico is unknown, because they are very poorly studied species.

The genus *Arion* Férussac 1819, the most diverse within the family Arionidae, is divided into four subgenera: *Arion* Férussac, 1819, *Kobeltia* Seibert, 1873, *Carinarion* Hesse, 1926 and *Mesarion* Hesse, 1926 (Breugelmans et al. 2013). The actual number of species worldwide is

unknown due to their wide polymorphism, which makes it difficult to identify many species (Quinteiro et al. 2005). About 25–50 nominate species exist (Barker 1999; Pincheel et al. 2005), suggesting that the taxonomic status of some of them should be confirmed combining molecular and morphological evidence such as in other groups of slugs (Nitz et al. 2009; Reise et al. 2011; Vendetti et al. 2019).

Several species of *Arion* have expanded their distribution range within the past century. For example, at least seven species have invaded other regions into Europe: *A. distinctus* J. Mabilie, 1868; *A. fasciatus* (Nilsson, 1823); *A. flagellus* Collinge, 1893; *A. hortensis* A. Férussac, 1819; *A. rufus* (Linnaeus, 1758); *A. silvaticus* Lohmander, 1937 and *A. vulgaris* Moquin-Tandon, 1855 (Hulme 2009). Other *Arion* species have reached other continents as a consequence mainly of globalization, and where the unintentional introduction of eggs, immature individuals or adults have provided for a steady barrage of slugs via shipments of mushrooms, cut flowers, flower bulbs, horticultural plants and vegetables (Barr et al. 2009). European species have been recorded both in synanthropic and disturbed environments in North America. For example, *A. rufus* and *A. subfuscus* (Draparnaud, 1805) were recorded as pests in the USA and Canada (Robinson 1999; Forsyth 2004), *Arion (Carinarion) circumscriptus* Johnston, 1828 was reported in Mexico City, Mexico, and *Arion (Kobeltia) intermedius* Normand, 1852 recorded from the border with the USA (Naranjo-García and Castillo-Rodríguez 2017).

The distribution of *A. vulgaris* includes Spain, Portugal, and the Azores (Zajac et al. 2017), and recently was reported in USA and Canada (Zemanova et al. 2018); although the native range is unknown. This species is commonly confused with *A. lusitanicus*, however, Quinteiro et al. (2005) used molecular markers to demonstrate that this species is restricted to the north-west region of the Iberian Peninsula. *A. vulgaris sensu stricto* is considered to be an invasive species which is widely distributed across Europe, which may have a negative impact on native slugs (Hulme 2009; Zemanova et al. 2017). In addition, this species inflicts damage to domestic gardens, vegetables, strawberry cultures, and cereal fields (Davies 1987; von Proschwitz 1997); and it is an intermediate host for several lungworm species that cause respiratory or systemic disease in canids and felids (Penagos-Tabares et al. 2020).

Arion intermedius is naturally distributed in North-Western Europe, and it has been introduced recently in Austria (Duda et al. 2019), North and South Africa (Connolly 1912; Cowie 1997), North and South America (USA, Chile and Colombia) (Hausdorf 2002; Cádiz and Gallardo 2007; Mc Donnell et al. 2008), Australia and New Zealand (Barker 1999). The presence of this species worldwide can be explained by its adaptability to different environmental conditions, its mixed breeding strategy that allows the production of viable offspring by single specimens (Jordaens et al. 2013),

Table 1. Species, sampling sites, habitats and catalogue number/specimens deposited (CNMO).

Species	Sampling sites	Habitat	CNMO/N
<i>Arion intermedius</i>	Hidalgo, Pueblo Nuevo. 20°08'43.6"; 98°41'05.5"W (HPN)	Pine-oak forest	8043 / 3
<i>A. intermedius</i>	Tlaxcala, Yauhquemecan, Cascadas de Atlhuetzia. 19°21'53"N; 98°10'57"W (TLX)	Under rocks, surrounding habitat buildings (hotel)	8044 / 13
<i>A. intermedius</i>	CDMX, Bosque de Tlalpan. 19°17'38.8"N; 99°12'15.9"W (BT)	Oak forest-xerophytic scrub	8045 / 7
<i>A. intermedius</i>	Puebla, Teopancingo, Protected Natural Area (Zona Protectora Forestal Vedada, Cuenca Hidrográfica del Río Necaxa). 20°06'96.6"N; 98°04'49"W (TE)	Pine-oak forest	8046 / 11
<i>A. intermedius</i>	Puebla, Venta Grande. 20°07'51.68"N; 98°05'21.63"W (VG)	Pine-oak and <i>Abies</i> forest	8047 / 9
<i>A. intermedius</i>	CDMX, Parque Nacional Desierto de los Leones. 19°19'32.96"N; 99°18'30"W (DLIB)	Coniferous forest	3470/ 12
<i>A. intermedius</i>	CDMX, Parque Nacional Desierto de los Leones, lago de los patos. 19°18'41.8"N; 99°18'34.33"W (DL)	Coniferous forest	8048/ 2
<i>A. vulgaris</i>	CDMX, Parque Nacional Fuentes Brotantes. (Mxd, Mx81, Mx82)	Secondary vegetation	8049-50/ 3

and the intensive commercial exchange between countries. No effect of *A. intermedius* has been documented on native populations, and it has been regarded as a pest only rarely. Although Barker (2002) reports effects on plant productivity in pastures of New Zealand and Cádiz and Gallardo (2007) suggested that this species can have negative effects on biodiversity by displacing native species from their habitats.

Introduced species act as vectors for parasites like rat lungworm, flukes, and nematodes that may impact native species, crops, pets, livestock and human health. In addition, given these species are potential pests, and a threat for native species; their populations should be monitored and controlled frequently. Hence, we report the presence of *Arion vulgaris* Moquin-Tandon, 1855 (*Arion lusitanicus* auct. non-Mabille) in Mexico for the first time, along with new records from *A. intermedius*, which was previously known from one report.

Materials and methods

Between 2014–2018 we surveyed 20 locations in Mexico, *Arion* species were collected in eight of these sites. Specimens were collected by hand after visual inspection under logs, rocks, stones, on vegetation, in cracks and between rocks. These animals were drowned and fixed with 70% ethanol. In addition, some samples were obtained from the Colección Nacional de Moluscos (CNMO) Instituto de Biología, Mexico City, Mexico (Table 1). The taxonomic identification was performed based on Maximum-likelihood phylogenetic analysis using two molecular markers: Nicotinamide adenine dinucleotide dehydrogenase 1 (ND1) and cytochrome oxidase 1 (COI) of mtDNA. In addition, external and internal morphological characters of these European species were used to confirm their taxonomic identity (Barker 1999; Cádiz and Gallardo 2007; Hatteland et al. 2015). All specimens analyzed were deposited in the CNMO catalog numbers (8043–8050).

Deoxyribonucleic acid (DNA) samples from 10 specimens (Table 1) were obtained of a foot fragment with the DNeasy Tissue kit (QIAGEN, Valencia, California, USA) after adhering to the manufacturer's instructions. DNA

amplifications were performed with primers for the mitochondrial ND1 gene (Quinteiro et al. 2005) and standard barcoding primers LCO1490 and HCO2198 for the COI (Folmer et al. 1994). Subsequently, amplifications reactions were carried out in a total volume of 25 μ l, containing 2.5 μ l of 10X PCR buffer, 2.5 mM MgCl₂ 10 mM of each deoxynucleotide triphosphate (dNTP), 10 pmol of each primer, 1U/ μ l Taq DNA polymerase (Invitrogen, CA, USA) and 1 μ l of template DNA. PCR-conditions were: an initial denaturation step at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s. Thereafter, DNA fragments were purified with QIAquick PCR purification kit (Qiagen, Valencia, CA), sequenced by Macrogen Inc., Korea and sequences deposited in the GenBank (access number: MN190030-32 COI and MN840825-27 ND1).

Molecular analysis

Each gene was aligned independently together with reference sequences obtained from GenBank with Muscle in Seaview ver. 4.0 (Gouy et al. 2010). Sequences were manually edited there to achieve positional homology. The fragment lengths after editing were 361 bp (ND1) and 514 bp (COI). In order to assign Mexican specimens to known *Arion* species, we performed Maximum-Likelihood (ML) phylogenetic analyses in PhyML ver. 3.0 (Guindon et al. 2010). In the case of *A. vulgaris*, we compared concatenated ND1 and COI sequences both of our specimens and those generated and deposited in the GenBank by Zemanova et al. (2016, 2018) for European individuals. From this analysis we exclude *A. intermedius*, because the COI and ND1 sequences of *A. intermedius* in the GenBank are not of the same specimen. The nucleotide substitution model that best fit our data set was determined in jModeltest ver 2.1.7 following the Akaike information criterion (AIC) (Posada 2008), it was HKY + G model with a gamma shape = 0.480. The *Arion flagellus* sequence (ND1: AY316247; COI: AY987880) was used as ingroup following to Zemanova et al. (2018). For the analysis of *A. intermedius* only ND1 sequences were used, we included in this analysis 46 *Arion* spp. sequences employed by Quinteiro et al. (2005), those deposited of this genera in the Genbank, and our own sequences of Mexican specimens. The nucleotide substitution model used was GTR + I + G with a gamma shape = 0.918, which also was determined with JModeltest following the AIC. The *Deroceras* sp. sequence (AY316292) was used as outgroup. In both analyses, the reliability of each node was tested using a bootstrap test after after 1000 pseudoreplicates.

Results

Molecular analysis

Of the ten Mexican specimens analyzed, three of them had ND1 sequences whose identity was > 94% with the *A. vulgaris* sequences deposited in GenBank,

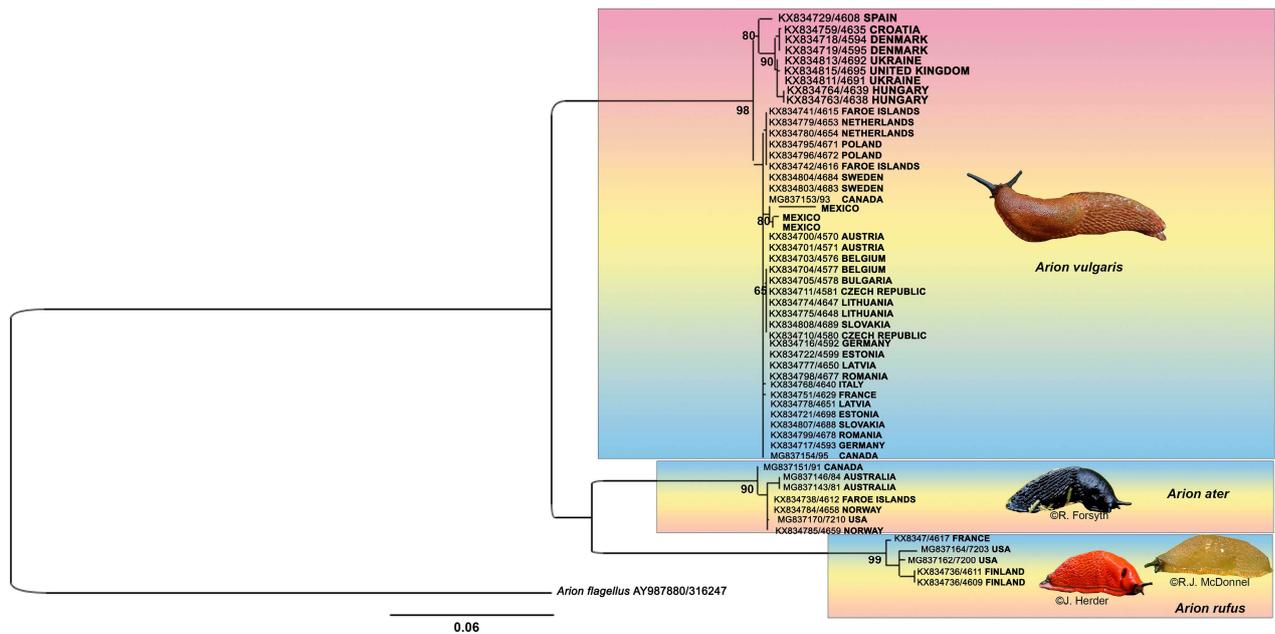


Figure 1. Maximum-likelihood phylogenetic analysis of *Arion vulgaris* based on 514 bp of COI gene and 361 bp of ND1 gene, using as the model best of nucleotide evolution HKY + G *sensu* Akaike criteria ($-\ln 3183.12842$, gamma shape = 0.480). Bootstrap values > 50% at nodes.

and the sequences of the rest of the specimens an identity > 98% with *A. intermedius*. Similarly, COI sequences of the three specimens had an identity > 99% with sequences of *A. vulgaris* from GenBank, while sequences of seven specimens had an identity > 95% with *A. intermedius*.

The phylogenetic analysis performed with concatenated genes clustered the three Mexican specimens with sequences of *A. vulgaris* from Canada, Faroe Islands, Netherlands, Poland, Sweden, Austria, Belgium, Czech Republic, Lithuania, Germany, Estonia, Latvia, Romania, Italy, France and Slovakia, while other group was formed by specimens from Spain, Croatia, Denmark, Ukraine, Hungary and UK with a bootstrap value > 50%. This *A. vulgaris* group was independent to those of *A. rufus* and *A. ater* (Figure 1). On the other hand, the phylogenetic analysis with ND1 clustered the sequences of Mexican specimens with the corresponding sequences of *A. intermedius* from Spain and UK, with a bootstrap value of 99 %. This species was clearly segregated from others *Arion* species included in the analysis (Figure 2). This analysis highlights that sequences of *A. vulgaris* specimens from Mexico were also clustered consistently with sequences of this species from Slovakia, Austria, France, Poland, and Canada (Figure 2).

Morphology

Arion vulgaris Moquin-Tandon, 1855

(Figure 3)

Live specimens (Figure 3) measured approximately 100 mm in length, (*A. vulgaris* shares the length of about 120 to 150 mm with *A. rufus* and *A. ater* (Pilsbry 1948; Zemanova et al. 2018)), were large and bulky with

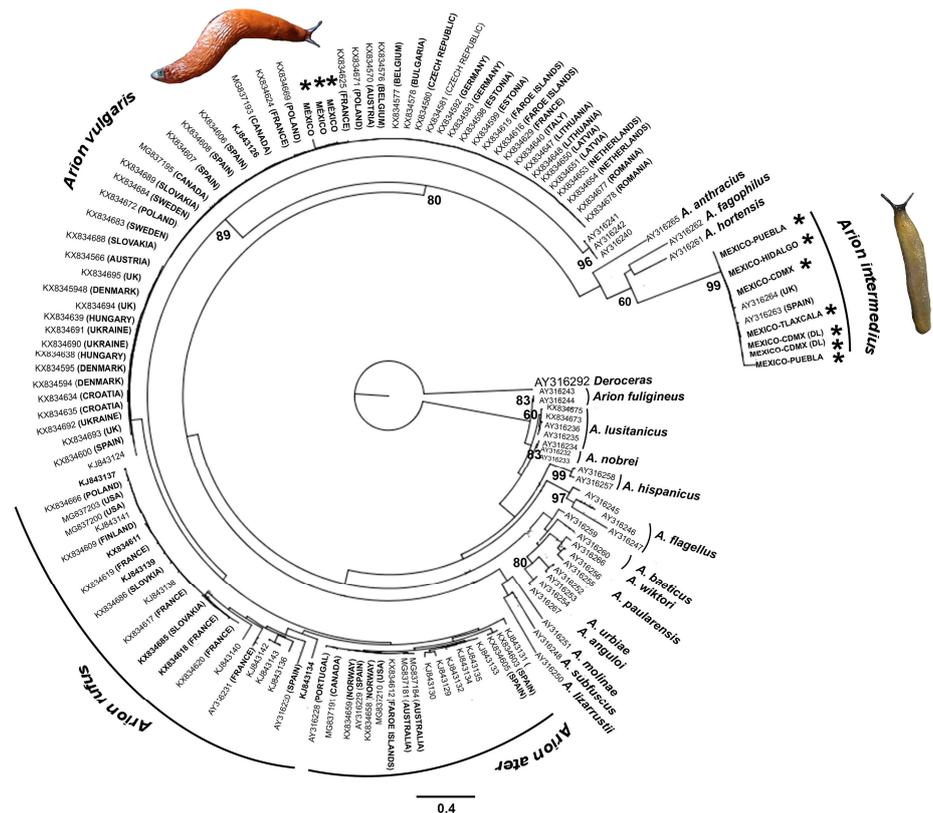


Figure 2. Maximum-likelihood phylogenetic analysis of *A. intermedius* based on 396 bp of ND1 gene, and using to the GTR+I+G as the model best of nucleotide substitution ($-\ln -8376.99268$, $I = 0.145$, gamma shape = 0.918) *sensu* Akaike criteria. Bootstrap values > 50% at nodes.

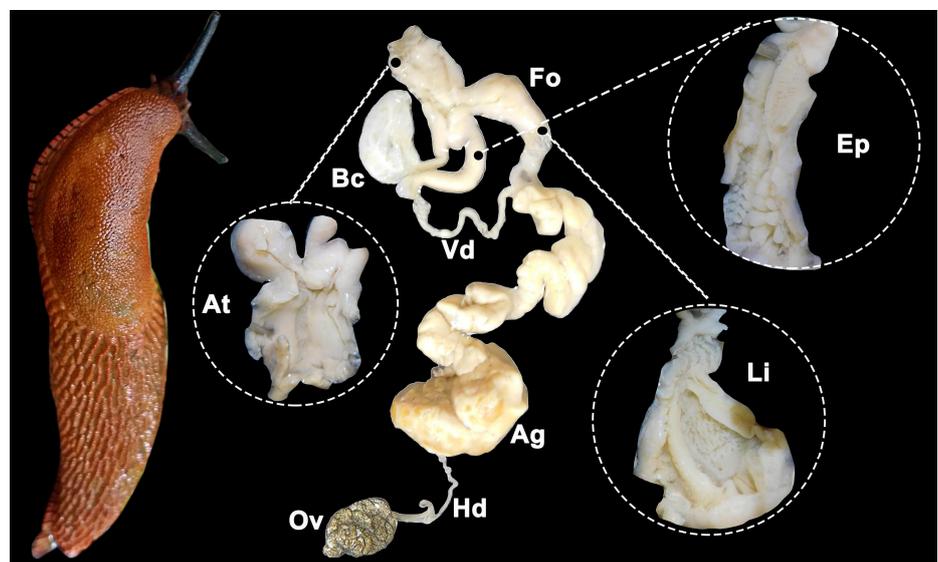


Figure 3. Specimen of *Arion vulgaris* and reproductive system showing internal anatomy of the inner wall of the At: Atrium, Ep: Epiphallus and Fo: Free oviduct. Ag: Albumen gland, Bc: Bursa copulatrix, Hd: Hermaphroditic duct, Li: Ligula, Vd: Vas deferens, Ov: Ovotestis. Photo by V. Araiza.

coarse tubercles on the back as well as on the sides. The color of their body was orange with a caudal mucus gland. The color of the sole is reddish, with small parallel blackish lines along of the sole; color pattern similar to that observed in species *A. rufus* and *A. ater*: cinnamon-brown to snuff brown

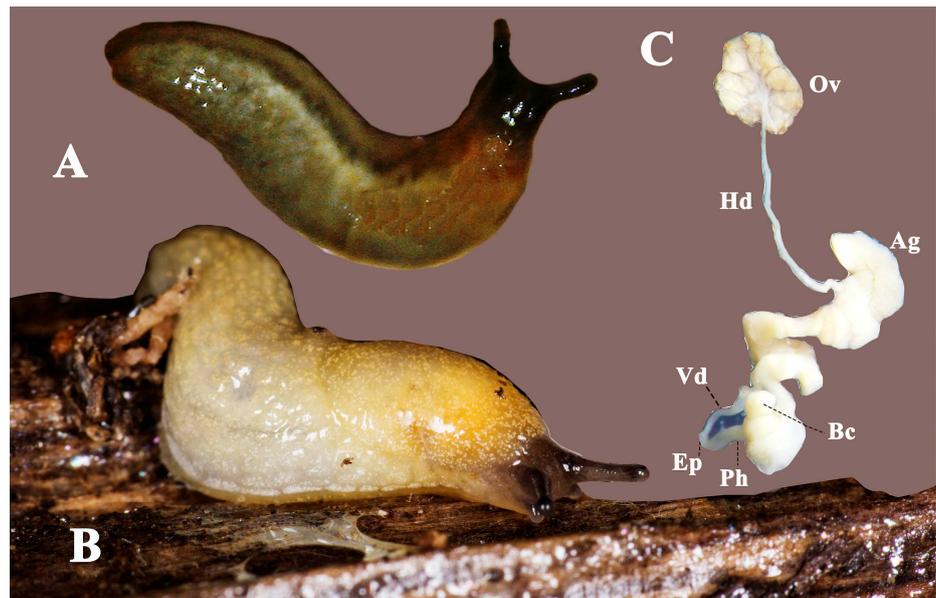


Figure 4. Live specimens of *Arion intermedius*. Specimen from Teopancingo (A), specimen from Bosque de Tlalpan (B), reproductive system (C), Ag: Albumen gland, Bc: Bursa copulatrix, Ep: Epiphallus, Hd: Hermaphroditic duct, Ov: Ovotestis, Ph: Phallus, Vd: Vas deferens. Photo by V. Araiza

(Pilsbry 1948). The head was a little darker than the rest of the body, whereas tentacles were almost black in color.

The morphology of the genitalia of three specimens is characterised by a short atrium; a slender epiphallus as long as the free oviduct; short bursa copulatrix duct; bursa copulatrix big and spherical; the free oviduct bulky; the pear-shaped ligula located inside the oviduct; the thin ligule, pointed at its distal end, and three times larger than that of the oviduct (Figure 3).

***Arion intermedius* (Normand, 1852)**

(Figure 4)

Live specimens measured approximately 18–25 mm in length, and slender when active. Body bearing pointed tubercles, which gives retracted individuals a prickly appearance. The body's color was yellowish, the specimens from Teopancingo had two faint dark lines on the sides (Figure 4A), whereas the rest lacked these lines (Figure 4B). All the specimens with a weakly defined gray border on the foot. The color of the head and tentacles was dark grey, whereas the specimens from Teopancingo were darker in color (almost black). The posterior of the dorsal surface rounded without a keel.

Genitalia with a short atrium and bulky; short phallus with a slender epiphallus; inner wall of the phallus nodulate; slender vas deferens as long as the epiphallus plus the phallus; the bursa copulatrix duct short and stout, its distal part forming a thickening ring; bursa copulatrix frequently small and spheroidal – only two of the thirteen specimens dissected had a large bursa copulatrix; very short vagina and stout; the oviduct free short and lacking internal ligula (Figure 4C).

Discussion

It is known that the external appearance of the genitalia is extremely similar with only subtle differences between *A. ater*, *A. rufus*, *A. vulgaris* and *A. lusitanicus*. However, several authors have proposed that the differences in size and shape of the ligula could be a diagnostic attribute for these species (Roth et al. 2012; Păpureanu et al. 2014; Hatteland et al. 2015). Previous molecular studies have demonstrated that these taxa can be correctly identified combining genetic data and genitalia morphology. Our phylogenetic analysis combining NDI and COI genes, as well as with NDI alone, clustered our specimens into the *A. vulgaris* group. In addition, the presence of a slender ligula pointed at the distal end located within the free oviduct in our specimens confirmed the identity of this species; however, its taxonomic usefulness should be evaluated in future studies considering both intra- and inter-specific variation within the genus.

Based on these results, this is the first record of *Arion vulgaris* in Mexico, and only the third for North America. The other records include an unconfirmed record in the USA (CABI: <http://www.cabi.org/isc/datasheet/6963>), and the other from Canada (Zemanova et al. 2018). Considering the invasive capacity of *A. vulgaris*, the high density of its populations, its rapid dispersion, and its role as an intermediate host of some bacteria (e.g., *Listeria monocytogenes*) and metastrongyloid lungworms (e.g., *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus*, *Troglostrongylus brevior* (Gismervik et al. 2015; Penagos-Tabares et al. 2020), this species might be a threat to health of wild and domestic animals in North America (Gismervik et al. 2015). Therefore, preventive management is necessary to avoid the transport and introduction of this slug into other locations via horticultural plants (Bergey et al. 2014).

We also report the expansion of distribution range of *Arion intermedius*, documenting the presence of this species in seven locations of Mexico's central region. Only one previous record was known of this species in Mexico from Parque Nacional Desierto de Los Leones, La Venta near Convento (Naranjo-García and Castillo-Rodríguez 2017). We demonstrate that the distribution of this species in Mexico is much broader. Perhaps the rapid expansion in the distribution of this species may be linked to extensive trade of plants that exists in this region of the country.

Our phylogenetic analysis clustered specimens of *A. intermedius* in a separated group from other *Arion* species. This clear differentiation might be attributed to its preferential self-fertilizing reproductive system, as has been suggested by some authors (Quinteiro et al. 2005). In Mexico, all development stages (adults, juveniles and eggs) have been recognized in the field, and are frequently associated in sympatry with *Deroceras laeve* (Müller, 1774) and non-native slugs: *D. reticulatum* (Müller, 1774), *D. invadens* (Reise et al., 2011), *Limax maximus* Linnaeus, 1758 and *Boettgerilla pallens* Simroth, 1912.

Even though many *Arion* species have been detected throughout the world in synanthropic as well as other disturbed environments (Cádiz and Gallardo 2007; Dedov et al. 2017; Reise et al. 2018; Vendetti et al. 2018), their spread poses a challenge for native slugs in undisturbed natural environments, as has been documented by Zemanova et al. (2017). Other studies as in Terra Nova National Park, Canada report non-native taxa in a high percentage in natural environments (Moss and Hermanutz 2010). Lastly, several plant nurseries are located near the sites reported in this study. Horticulture may play a role in passive dispersal of non-native species and immediate actions such as monitoring and eradication are required to prevent further dispersal of these *Arion* species and other invasive species to other regions in Mexico.

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