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Molecular and taxonomic characterisation of introduced specimens of *Poecilia reticulata* in the lower Paraguay River basin (Cyprinodontiformes: Poeciliidae)

Juan José Rosso[1,2,3], Franco del Rosso[3,4], Ezequiel Mabragaña[1,2,3], Nahuel F. Schenone[5], Esteban Avigliano[2,3,6] and Juan Martín Díaz de Astarloa[1,2]

Poeciliids comprise around 300 species inhabiting the fresh and brackish waters of the Americas and Africa. *Poecilia reticulata* is native to Northeastern South America and Trinidad and Tobago. In this paper, introduced specimens of *P. reticulata* collected in the lower Paraguay River in Argentina, were characterized by means of molecular and taxonomic approaches. We further explore, by means of DNA Barcoding, the singularity of the genetic identity of these specimens. Ocurrence of *P. reticulata* in the lower Paraguay River represents the first record of this species in Argentina. Thirteen individuals of *P. reticulata* were collected. DNA barcoding showed that all five specimens sampled belong to a single mitochondrial lineage, which was also present in 11 countries from five continents. The distance-based tree clearly grouped separetely four different clusters of *P. reticulata* when including public data. Genetic distance between the most divergent *P. reticulata* almost paralleled distance between this species and *Poecilia mexicana* and *P. vivipara*. Established populations from Paraguay could be one of the plausible sources for the introduced populations recorded in the lower Paraguay River. The presence of *P. reticulata* in an open waterway with known drainage to a natural stream is of major concern.

Keywords: Argentina, DNA Barcoding, Guppy, *Poecilia*, Taxonomy.

Poecilídeos compreendem cerca de 300 espécies que habitam águas doces e salobras das Américas e África. *Poecilia reticulata* é nativa do nordeste da América do Sul e Trinidad e Tobago. Neste trabalho, espécimes introduzidos de *P. reticulata* coletados no baixo rio Paraguai na Argentina, foram caracterizados por meio de abordagens moleculares e taxonômicas. Exploramos ainda, por meio de DNA Barcoding, a singularidade da identidade genética destes espécimes. A ocorrência de *P. reticulata* no baixo rio Paraguai representa o primeiro registro dessa especie na Argentina. Treze indivíduos de *P. reticulata* foram coletados. O Barcoding mostrou que todos os espécimes pertencem a uma única linhagem mitocondrial, a qual está presente em 11 países dos cinco continentes. A árvore de distâncias agrupou separadamente quatro clusters diferentes de *P. reticulata* quando incluindo dados públicos. A distância genética entre os agrupamentos mais divergentes de *P. reticulata* quase igualou a distância entre esta espécie e *Poecilia mexicana* e *P. vivipara*. As populações estabelecidas no Paraguai poderiam ser uma das fontes plausíveis para as populações introduzidas registradas no baixo rio Paraguai. A presença de *P. reticulata* em um canal aberto com drenagem conhecida para um córrego natural é de grande preocupação.

Palavras-chave: Argentina, DNA Barcoding, Guppy, *Poecilia*, Taxonomia.

[1]Grupo de Biotaxonomía Morfológica y Molecular de Peces, Instituto de Investigaciones Marinas y Costeras (IIMyC-CONICET), Universidad Nacional de Mar del Plata, Funes 3350, 7600 Mar del Plata, Buenos Aires, Argentina. (JJR) plurosso@yahoo.com.ar (corresponding author), (EM) emabraga@mdp.edu.ar, (JMDA) astarloa@mdp.edu.ar
[2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290, C1425FQB Buenos Aires, Argentina.
[3]Fundación Bosques Nativos Argentinos para la Biodiversidad, Colombres 962 Piso 3A, C1218ABL Buenos Aires, Argentina.
[4]Ministerio de la Producción y Ambiente de la Provincia de Formosa, Belgrano 878, 6th floor, 3600 Formosa, Argentina. fradelrosso@hotmail.com
[5]Centro de Investigaciones Antonia Ramos (CIAR), Acceso Balneario Bonito s/n, 3361 Villa Bonita, Campo Ramón, Departamento de Oberá, Misiones, Argentina. nahuelschenone@hotmail.com
[6]Instituto de Investigaciones en Producción Animal (INPA-CONICET), Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Av. Chorroarín 280, C1427CWO Buenos Aires, Argentina. crotalus1451@yahoo.com.ar

Introduction

Poeciliids are small ovoviviparous fish that thrive in small, often ephemeral, freshwater environments. Their high reproduction rate is the biological key to thrive quickly in these ephemeral aquatic environments with just a small temporal window of favorable environmental conditions (Meffe, Snelson, 1989). Due to their high capacity to reproduce in captivity, poeciliids have become one of the most widespread species in the aquarium trade and are also commonly used for experimental studies in biology (Magurran, 2005).

The guppy, *Poecilia reticulata* Peters, 1859, is native of Northeastern South America and Trinidad and Tobago, with the type locality in the Guayre River in Venezuela. Native range of this species includes Brazil, Guyana, Venezuela, and Trinidad and Tobago (Rosen, Bayley, 1963). Currently, its presence in natural environments is known to occur in more than 69 countries outside of the species native range (Deacon *et al.*, 2011). Known introduced range includes Asia, Australasia-Pacific, Europe, North America, and South America (GBIF, 2016). In South America, poecillids have been recorded mostly in the northern region of the continent. Just recently, by means of voucher examination from several Museums worldwide, the presence of this invasive species was confirmed in Paraguay (Lucinda, 2017).

Multiple introductions of guppies worldwide are likely to have occurred for mosquito control and release from domestic aquariums. There are official records of its introduction for mosquito control in Australia (Vipan, 1910) and in California swamps of United States (Shapovalov *et al.*, 1981; Dill, Cordone, 1997). In Australia, wild populations were established before the 1970s (Allen *et al.*, 2002). In the United States, *P. reticulata* is considered a hazard to native cyprinids and killifishes and has been implicated in the decline of native fishes in the southwest (Courtenay, Stauffer, 1990). It is also a known carrier of trematode parasites, which may affect native fish populations (Nico, Martin, 2001).

Several approaches to evaluate genetic divergence among different populations have been carried out in order to relate these findings with contrasting life history traits (Reznick, 1982; Reznick, Bryga, 1987) and invasion success (Lindholm *et al.*, 2005). Genetic differentiation was also interpreted as being the consequence of independent cases of incipient speciation in *P. reticulata* (Alexander, Breden, 2004; Russell, Magurran, 2006). Indeed, two new species, formerly regarded as populations of *P. reticulata*, were recently described (Poeser *et al.*, 2005; Schories *et al.*, 2009). Molecular phylogenetic analyses, finally revealed that these three guppy species are old species, which arose by parallel evolution rather than being emerging new species (Schories *et al.*, 2009). Therefore, a proper taxonomic identification and molecular characterisation of introduced populations of the invasive *P. reticulata* is necessary. Similarly, color polymorphisms in males should also be reported for each locality since they play a central role in female mate choice (Godin, Dugatkin, 1996) finally leading to strong social influences on female generalized and repeatable trait-copying behaviour (Godin *et al.*, 2005). A conspicuously colored male is also a male that presents improved behavior traits (boldness and escape distance) against fish predators (Godin, Dugatkin, 1996). Therefore, female choosing such a male would gain these indirect fitness benefits. Color patterns are also relevant to evade or confuse visually oriented predators (Endler, 1978). Ultimately, the spatial distribution of melanophores, is decisive for the differentiation between *P. reticulata* and *P. wingei* Poeser, Kempkes & Isbrücker, 2005 (Poeser *et al.*, 2005).

The objective of this paper is to characterize introduced specimens of *P. reticulata* collected in the lower Paraguay River drainage by means of molecular and taxonomic approaches. We further explore the genetic identity and singularity of these specimens by means of DNA Barcoding.

Materials and Methods

Study Area. The Paraguay River extends 2,670 km southwards from its source in the western hills of the Brazilian Shield to its confluence with the Paraná River, encompassing an area of more than 1 million of km^2 draining areas of Brazil, Bolivia, Paraguay and Argentina. The Paraguay River is scarcely disturbed by human activities and fisheries are still at a low level of exploitation when compared with the Paraná and Uruguay rivers (Quirós *et al.*, 2007). The meridional section of the Paraguay River between the Apa River and the confluence with the Paraná River represents the Lower Paraguay (Neiff, 1990). Introduced specimens of *P. reticulata* included in our analyses were collected in a restricted biotope (26° 11' 52" and 58° 13' 1.5") of the lower Paraguay River basin, in the province of Formosa, Argentina. Collecting site averaged a depth of 48 cm and its largest wetted width was 1.6 m. Water velocity at time of sampling was almost nil. Instream macrophytes were represented by submersed and floating forms that hardly cover more than 30% in a stream reach of 50 m. Bottom was dominated by soft sediments and fish cover was limited to macrophytes mats. This channel tributes its waters to the Pucú stream, a natural tributary of the lower Paraguay River (Fig. 1).

Fish sampling and taxonomy. Fishes were collected with hand and seine nets during a sampling program of the lower Paraguay River fish fauna. Individuals were identified with the taxonomic literature available (Rush Miller *et al.*, 2010), measured and sexed. Meristic and basic morphometrics were obtained from specimens after fixation. Meristic counts included total fin rays, and the number of scales in predorsal, lateral, circumpeduncular and transverse series. Basic morphometrics were obtained with a digital caliper under 10X augmentation from point to point. Color pattern in males was described following Kottler *et al.* (2013).

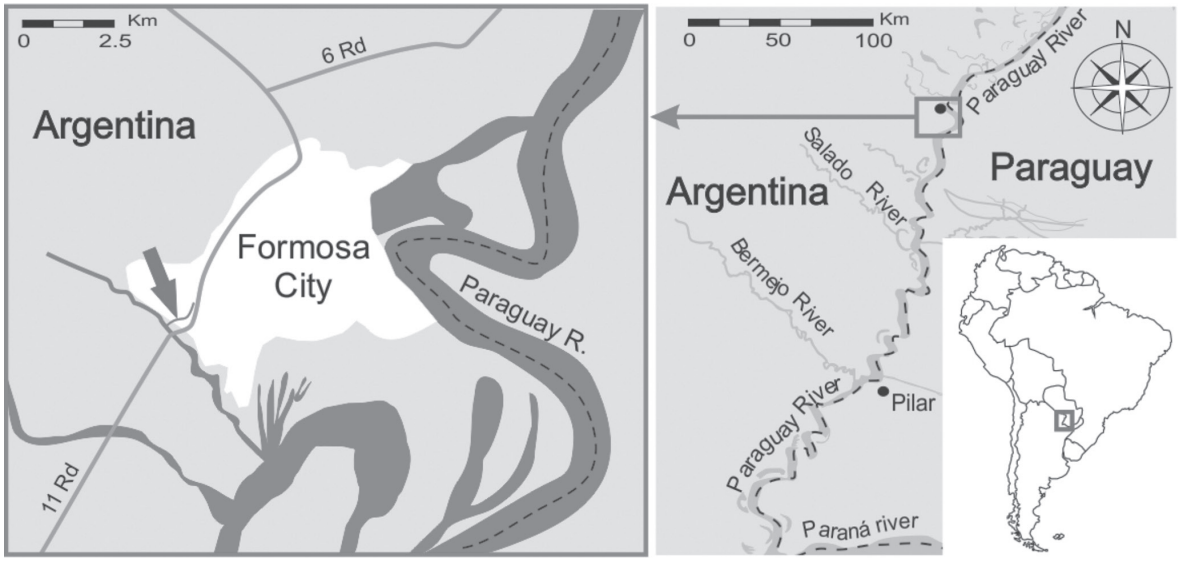

Fig. 1. Map of the study area showing the exact location of the collecting site.

Molecular analysis. A total of five individuals were subsampled in order to extract a piece of white muscle for molecular analysis. For each individual a segment of the mitocondrial DNA corresponding to the 5' region of COI was sequenced. Standard DNA barcoding protocols (Ivanova *et al.*, 2006) were followed for DNA extraction, polymerase chain reaction (PCR) and sequencing. Extraction, PCR and sequencing protocols are described in Rosso *et al.* (2012). For each sequence, the Barcode Index Number (BIN) was reported. The Barcode of Life DataSystem (BOLD) was explored for additional sequences and BINs of *P. reticulata* in order to seek for an agreement at a specific level. Sequence divergences among BINs and their geographic distributions around the world were reported. Sequence divergences were calculated using the Kimura two parameter (K2P) distance model (Kimura, 1980). A neighbour-joining (NJ) tree of K2P distances was constructed to displayed genetic divergences. *Poecilia vivipara* Bloch & Schneider, 1801 (the type species of the genus and the only former species of this genus reported for Argentina) and *Poecilia mexicana* Steindachner, 1863 were included as outgroups.

Results

A total of 13 individuals of *P. reticulata* were collected. No other accompanying fishes were present at time of sampling. Voucher specimens were deposited in the fish collection of the Instituto de Investigaciones Marinas y Costeras (UNMDP-CONICET) in Mar del Plata, Argentina. Morphometrics of collected specimens are displayed in Tab. 1. Total dorsal-fin rays 7 (n=13), total pectoral-fin rays 14 (n=11), 15 (n=2), total pelvic-fin rays 6 (n=13), total anal-fin rays 8 (all males), 9 (all females), branched caudal-fin rays 12 (n=1), 13 (n=10), 14 (n=1). Predorsal scales 12 (n=5), 13 (n=7), longitudinal scales 25 (n=4), 26 (n=4), 27 (n=5), transverse series 6 (n=4), 7 (n=9), scales around caudal peduncle 13 (n=1), 14 (n=11), 15 (n=1).

Some females were found with macroscopic signs of advanced incubation of embryos. Males, as usual, showed variability in coloration patterns whereas females displayed a plain gray-silvery background. Color traits in males displayed the characteristic black spots and orange blotches (Fig. 2).

Neither anterior or posterior black horizontal stripes nor ventral black lining of caudal peduncle or black pigment on dorsal fin were observed. None of the collected males showed the characteristic blue iridescent spot of wild males. However, the caudal-black spot was surrounded by a blue ring in some males. Caudal fin did not displayed the orange-black lining nor the black spot on ventral lobe. Conversely, caudal fin displayed different combinations of concentrated melanophores, black or orange spots and white or orange stripes.

Tab. 1. Morphometrics of collected specimens of *Poecilia reticulata* from lower Paraguay River basin, Formosa, Argentina. Values 1-8 are percentages of the standard length and values 9-11 are percentages of head length.

	Males (n=7)	Females (n=6)
Standard Length (mm)	14.3-16.9	17.2-22.1
1. Head length	26.9-29.7	25.4-29.9
2. Predorsal distance	54.4-59.7	54.8-64.5
3. Dorsal-fin base length	8.99-10.8	8.1-10
4. Anal-fin base length	6.42-8.63	7.2-8.8
5. Body depth	21.8-24.3	22.6-24.3
6. Pre-pelvic distance	40.7-44.6	43.6-50.1
7. Pre-anal distance	46.9-52.1	59.3-63.3
8. Caudal peduncle depth	17.6-19.5	15.1-17.2
9. Snout length	16.1-25.9	23.7-29.5
10. Orbital diameter	31.7-37.2	30.8-34.9
11. Postorbital length	40.7-46.8	40-51.9

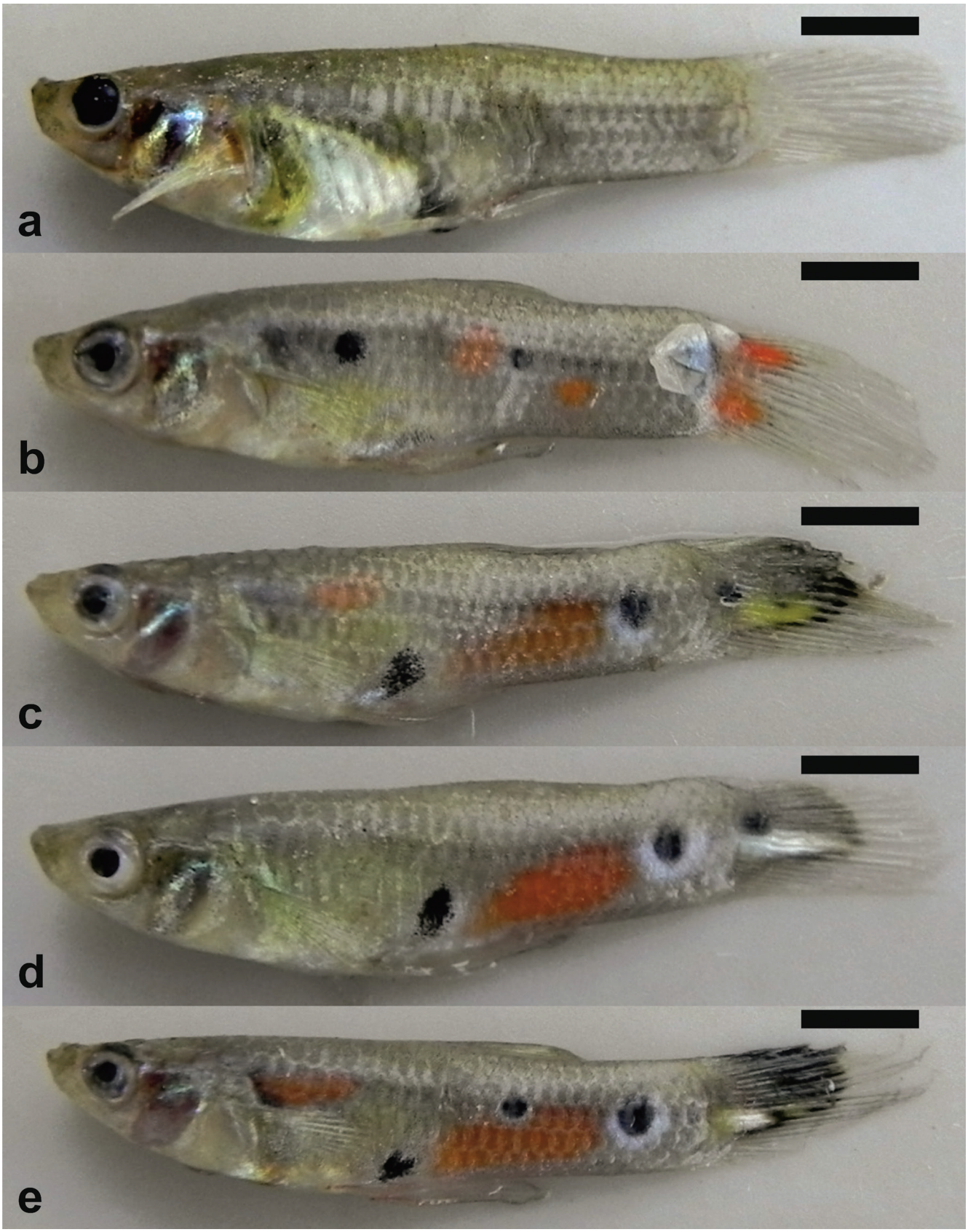

Fig. 2. Selected collected specimens of *Poecilia reticulata* in the lower Paraguay River. **a**. female: UNMDP 3687, 22.1 mm SL; **b**. male 1: UNMDP 3693, 15.9 mm SL; **c**. male 2: UNMDP 3694, 15.9 mm SL; **d**. male 3: UNMDP 3692, 16.9 mm SL; **e**. male 4: UNMDP 3691, 16.8 mm SL. Scale bars: 3 mm.

DNA Barcoding showed that all specimens from the lower Paraguay River belong to a single mitochondrial lineage as they were all grouped into a single BIN. This BIN, ACE3484, also contains specimens widely distributed worldwide, being detected in 11 countries of five continents (Tab. 2). Three additional BINs under the taxonomic denomination of *P. reticulata* were detected in BOLD. Mean and maximum intra-BIN genetic distances as well as distances to the nearest neighbours are also depicted in Tab. 2. The K2P NJ tree clearly grouped separately four different clusters of *P. reticulata* (Fig. 3). Nevertheless, the K2P distance was well over 2% only between BIN ACC0443 and the sister cluster composed by the three remaining BINs (Tab. 3). Among these three BINs, genetic distances ranged from 1.1 to 2.1 %.

Tab. 2. Geographic location and distance summary of public BIN for *Poecilia reticulata*. N: number of specimens; Min: minimum genetic distance whithin BIN; Max: maximum genetic distance whithin BIN; NND: nearest neighbour distance and NND BIN: Barcode Index Number of the nearest neighbour.

BIN	N	Occurrence	Min	Max	NND	NN BIN
ACE9037	10	Brazil	0.02	0.34	1.05	AAD1850
ACC0443	6	Indonesia	1	2.35	4.02	AAD1850
ACE3484	39	Argentina Brazil Trinidad and Tobago India Bangladesh Indonesia Australia Germany Kenya South Africa Cape Verde Nigeria	0.27	1.57	1.19	AAD1850

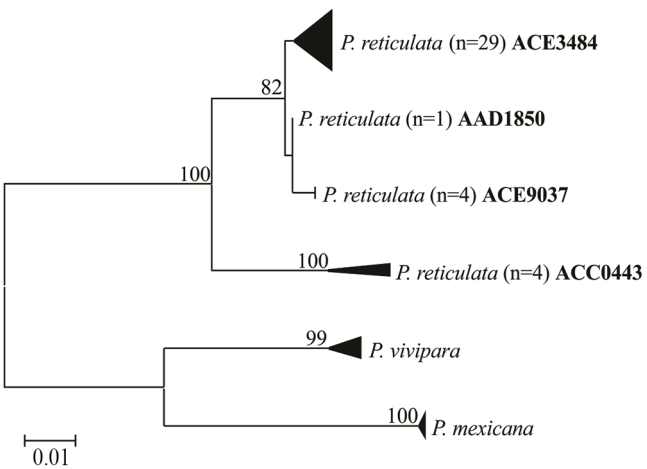

Fig. 3. NJ K2P tree of COI sequences of collected (ACE3484) specimens of *Poecilia reticulata*. The remaining sequences of this species together with outgroups were gathered from BOLD.

Discussion

Among the existing valid species, *P. wingei* and *P. obscura* Schories, Meyer & Schartl, 2009 more closely resemble *P. reticulata*. The dorsal fin count clearly distinguish *P. reticulata* (7) from the recently described *P. obscura* (6). Females of *P. wingei* are indistinguishable from those of *P. reticulata* (Poeser *et al.*, 2005). Instead, males of *P. reticulata* are larger and lack the conspicuous vertical band sometimes present in the midsection of the body, at the anterior part of the caudal peduncle of *P. wingei*.

The occurrence of *P. reticulata* in the lower Paraguay River basin represents the first record of this species for freshwater ecosystems of Argentina. This genus was recorded only once in natural environments of Argentina. The species reported was *P. vivipara* (Pozzi, 1945; Ringuelet, 1967). However, the presence of this species was never referred to collected and preserved specimens and therefore Koerber, Litz (2014) sugested not regarding this species as present in Argentina. Consequently, the record of *P. reticulata* in the lower Paraguay River basin, in Formosa province, also represents the first record of the genus in Argentina supported by preserved specimens.

The analysis of the global distribution of *P. reticulata* revealed that this species is now established in at least 69 countries outside of its native range (Deacon *et al.*, 2011). International online databases reported that *P. reticulata* was collected only once in the Paraguay River basin. The exact location (25° 10’ 12’’ S, 57° 30’ 0’’ W) is a small stream near Costa Azul town, 200 m from highway Asunción-Ciudad del Este (GBIF, 2016). Introduced populations in Paraguay are considered to be established (Deacon *et al.*, 2011). However, it was not until recently, that the presence of *P. reticulata* in this country was confirmed by inspection of voucher material deposited in museums (Lucinda, 2017). Most of the localities reported by this author are concentrated in the lower Paraguay River, near the border with Argentina. Therefore, this could be one of the plausible sources for the introduced populations recorded in the lower Paraguay River in Argentina. Alternatively, unscrupulous release from aquarists may also explain this finding. Irrespective of the source, the presence of *P. reticulata* in an open waterway with known connectivity to a natural stream is of major concern.

During simultaneous field trips in other river reaches of the lower Paraguay River basin, several specimens of two native Cyprinodontiformes (*Phallotorynus* Henn and *Cnesterodon* Garman) not previously recorded for this area were also collected. Naturally, the autoecology of these small-sized species in the lower Paraguay River is unknown. Consequently, little can be anticipated about the impact of a likely invasion of *P. reticulata* on natural populations of these species. Nevertheless, the dramatic and human-mediated range expansion of the invasive *P. reticulata* can negatively impact native freshwater communities (Deacon *et al.*, 2011). Therefore, possible interactions between *P. reticulata* and these native Cyprynodontiformes in the lower Paraguay River, could lead to a negative effect on native species if this invasive fish spreads further within this river network.

Tab. 3. Summary of genetic distances among BINs of *Poecilia reticulata* and both species of *Poecilia* utilized as outgroups. Mean: mean within group distance.

		N	Mean	Mean between group distance				
				AAD1850	ACC0443	ACE3484	ACE9037	AAC0279
P. reticulata	AAD1850	1						
P. reticulata	ACC0443	4	0.015	0.045				
P. reticulata	ACE3484	20	0.004	0.016	0.051			
P. reticulata	ACE9037	4	0.000	0.011	0.051	0.021		
P. vivipara	AAC0279	7	0.006	0.138	0.155	0.148	0.145	
P. mexicana	AAA4518	8	0.003	0.148	0.153	0.152	0.150	0.103

Our analysis of COI sequences evidenced a marked divergence among populations of *P. reticulata* around the world. Earlier studies on allozyme and DNA sequences also revealed a marked genetic differentiation among guppy populations (Carvalho *et al.*, 1991; Shaw *et al.*, 1991). Whether these genetic divergences match taxonomic divergences is a matter of debate. For some authors, this genetic differentiation is the consequence of independent cases of incipient speciation (Alexander, Breden, 2004; Russell, Magurran, 2006). Conversely, Poeser *et al.* (2005) considered this genetic divergence as a case of genetic differentiation without speciation.

In our study, the genetic divergence between the two most disparate BINs of *P. reticulata* almost paralleled genetic distances between this species and other species of the same genus (but different subgenus) as *P. mexicana* and *P. vivipara*. Therefore, the likely occurrence of a complex of species under the taxonomic denomination of *P. reticulata* should not be completely disregarded. Several other species of the genus *Poecilia* were erected as species complex. Particularly, *Poecilia sphenops* Valenciennes, 1846 was considered to be a species complex by Alda *et al.* (2013) and a recent revision of the phylogeny and biogeography of poeciliids (Ho *et al.*, 2016) also regarded *P. mexicana* and *Poecilia latipinna* (Lesueur, 1821) as species complexes.

Genetic divergences within *P. reticulata* could also be partially explained by misidentifications. Due to identical meristic counts and similar color patterns between females of *P. reticulata* and *P. wingei* (Poeser *et al.*, 2005), the possibility of females of *P. wingei* being mislabelled as *P. reticulata* by non-taxonomist researchers for some of the published BINs should not be disregarded. Nevertheless, this will only account for one (assuming a unique BIN for *P. wingei*) of the four BINs reported for *P. reticulata* worldwide and therefore, the hypothesis of a species complex is still valid. The specimens of the lower Paraguay River were unambiguously identified as *P. reticulata* and not *P. obscura* and *P. wingei* following dorsal fin counts and male color patterns respectively. Therefore, we provided the DNA Barcode library with a reference BIN for *P. reticulata* further supported by Museum vouchers. This certainly will aid in future analyses of introduced populations of *Poecilia* regarded as being *P. reticulata*. Irrespective of the genetic identity, a phylogenetic study revealed that these three species (*P. reticulata*, *P. wingei* and *P. obscura*) are different taxonomic units (Schories *et al.*, 2009).

Investigations in Trinidad (Reznick, 1982; Reznick, Bryga, 1987; Reznick *et al.*, 1990) demonstrated that guppies exposed to increasing levels of predation intensity on larger specimens mature at a smaller size, displayed a higher allocation of energy to reproduction and consequently reproduced more frequently with more and smaller offspring. The top predator in these fundational studies were fish of the genus *Crenicichla* (Cichlidae), a locally abundant genus in the lower Paraguay River basin. Therefore, it could be argued that *P. reticulata* already possesses the behavioural traits and life history adaptability to deal with this predator . Nevertheless, observed size range of males suggested that introduced specimens collected in the lower Paraguay River were not exposed yet to a intense risk of predation. This may be inferred, by the wider size range (suggestive of low risk of predation on larger specimens) observed in the lower Paraguay River, compared with the narrower size ranges reported for populations under high risk of predation living in natural streams (Reznick, Endler, 1982).

Due to ovoviviparity and sperm storage (Meffe, Snelson, 1989) it could be expected that the small number of "founders" collected will finally suceed into a established population. Indeed, experimental studies demonstrated that a single individual of *P. reticulata* can lead to flourishing populations of this invasive fish and called for caution when introducing this species to natural ecosystems (Carvalho *et al.*, 1996; Deacon *et al.*, 2011). Albeit equiped with these adaptive reproductive traits, founders must also face regulatory factors derived from interactions with other fishes, either competitors or predators.

The molecular and taxonomic characterisation of the introduced specimens of *P. reticulata* collected in the lower Paraguay River basin, certainly will aid in scrutinizing the identity of eventual future records of this exotic invasive species in the lower Plata River basin. So far, the detection of the first individuals may lead to timely management actions impairing the successful establishment of this invasive species. Of central importance it is also to further trace the original source of this introduced population as well as to explore its connectivity with nearby populations. For conservation purposes, the geographic occurrence and abundance distribution of this species within the lower Paraguay River should be mapped.

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