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Incipient genetic divergence or cryptic speciation? *Procamallanus* (Nematoda) in freshwater fishes (*Astyanax*)

Ana Santacruz[1,2,3] | **Claudia Patricia Ornelas-García**[1] | **Gerardo Pérez-Ponce de León**[1,3]

[1]Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico

[2]Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Mexico City, Mexico

[3]Escuela Nacional de Estudios Superiores unidad Mérida, Mérida, México

Correspondence
Gerardo Pérez-Ponce de León, Escuela Nacional de Estudios Superiores unidad Mérida, UNAM. Km. 4.5 Carretera Mérida-Tetiz, Ucú, Yucatán, México.
Email: ppdleon@enesmerida.unam.mx

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Abstract

Hosts provide the main environmental traits parasites have to deal with, resulting in covariation between both associates at both micro- and macro-evolutionary scales; phylogenetic analyses of highly host-specific parasites have shown that parasite and host phylogeny might be highly congruent, and adaptation of a host species to new environments may lead to concordant changes of their parasites. *Procamallanus* (*Spirocamallanus*) *neocaballeroi* is a highly host-specific parasitic nematode of the Neotropical freshwater fish genus *Astyanax* in Mexico. One of the host species of the nematode is the emblematic Mexican tetra, *A. mexicanus,* which exhibits two contrasting phenotypes, a cave-dwelling morph (with troglomorphic features), and the surface-dwelling morph; other congeneric species inhabit rivers and lakes, and some of them occur in sympatry, displaying trophic specializations. Here, we explored the hypothesis that contrasting environments (surface rivers vs cave rivers), and host morphological divergence (sympatric ecomorphs in a lacustrine environment) might result in the divergence of their parasites, even though the hosts maintain a cohesive genetic structure as the same species. To test the hypothesis, several populations of *Astyanax* spp. were sampled to search for *P.* (*S.*) *neocaballeroi*. The nematode was found in 10 of the 52 sampled sites; two localities corresponded to cave populations. The phylogenetic analysis based on COI sequences yielded three major lineages for *P.* (*S.*) *neocaballeroi*. We found no concordance between the three lineages and the habitat where they occur in *Astyanax mexicanus*, even considering those living in drastic environmental conditions (caves), or between these lineages and lacustrine ecomorphs of *Astyanax aeneus* and *A. caballeroi* occurring in sympatry. Instead, genetic lineages of the nematode exhibit a clearer pattern of host species association and geographical distribution; our results showed that *P.* (*S.*) *neocaballeroi* is experiencing an incipient divergence although the morphological study of lineages shows no conspicuous differences.

KEYWORDS

Astyanax, cryptic species, DNA, nematoda, species delimitation, taxonomy

1 | INTRODUCTION

Parasites are model systems to study adaptive radiations because they represent the most successful and widespread mode of life on earth (Price, 1980). A close association is established between parasites and their hosts; this implies that even subtle changes displayed by any of the members of the association may impact the evolutionary fate of the other associate, on a micro- or macro-evolutionary scale. Co-divergence patterns reflect simultaneous branching in the phylogeny of hosts and parasites (Martínez-Aquino, 2016); these patterns are the result of a complex relationship between historical biogeography and host and parasite life history traits (see Bothma et al., 2020 and references therein); however, diversification may occur through host switching events, duplication or lineage sorting/extinction, resulting in incongruence between the phylogeny of hosts and parasites (Johnson et al., 2003). At microevolutionary scale, the population genetic structure in host-specific parasites can be strongly mediated by the hosts traits and thus regulate parasite transmission (McCoy et al., 2003). The isolation of parasites by niche changes and host traits promote the divergence of their populations. In addition, the complexity of the parasite life cycle can determine the degree of genetic structure and the microgeographic adaptations; in parasites with relatively simple life cycle patterns, a stronger genetic structure is shown (Criscione & Blouin, 2004; Blasco-Costa & Poulin, 2013). Instead, in parasites with complex life cycles, the free-living stages are subject to selective pressures exerted by the environment and its host (Huyse, Poulin & Theron, 2005; Lymbery, 2015). Furthermore, highly vagile host species could maintain gene flow between distant populations, and parasite populations are less genetically structured (Blasco-Costa, Waters & Poulin, 2012).

With more than 147 species, *Astyanax* is the most diverse genus of freshwater fish in the Americas (Eschmeyer, 2014). In Mexico, eight species are considered valid according to Ornelas-García et al. (2008). Among them, the Mexican tetra, *A. mexicanus,* exhibits the most northern distribution range of the genus and was able to colonize an adapt to caves multiple times during their evolutionary history (Gross, 2012; Elliott, 2015), giving rise to a parallel evolution reflected in the drastic adaptive changes associated with the cave environment (Jeffery, 2012). Phenotypically, the Mexican tetras in caves are troglobitic, with morphological, physiological and behavioural changes, including lack of eyes and pigment loss as the most visible characters (Torres-Paz et al., 2018). Genetic evidence suggests that at least in one cave (Chica), an admixture occurs between individuals of *A. mexicanus* from surface and cave waters (Bradic et al., 2012; Strecker, Hausdorf & Wilkens, 2012; Herman et al., 2018). Another conspicuous example of parallel evolution under ecologically divergent scenarios within the genus is shown by the sympatric lacustrine species across Mexican and Central America tropical lakes, that is *A. aeneus* and *A. caballeroi*; *A. nasatus, A. nicaraguensis* and *A. bransfordii* (Ornelas-García et al., 2008; Powers et al., 2020). In Lake Catemaco, Mexico, the morphs of *A. aeneus* and *A. caballeroi* are highly divergent, and as a result, they were originally considered as different genera (*Astyanax* and '*Bramocharax'*); however, recent molecular studies have shown that they belong not only to the same genus, but also they readily hybridize and show no sign of genetic differentiation, although morphological and ecological differences are evident among *Astyanax* ecomorphs (Ornelas-García et al., 2018).

Individuals of *Astyanax* spp. are infected by a highly host-specific nematode, *Procamallanus* (*Spirocamallanus*) *neocaballeroi* across Mexico; this parasite species has only been found, as adult, parasitizing the intestine of some species of *Astyanax* in Middle America (Choudhury et al., 2017). The life cycle of *P.* (*S.*) *neocaballeroi* involves a copepod as intermediate host, and a fish as a definitive host, where the parasite matures and reproduces (Moravec & Vargas-Vázquez, 1996). In this study, we tested the hypothesis that the nematode *P.* (*S.*) *neocaballeroi* may represent a species complex corresponding with the habitat where their host species live (surface vs caves), or with the morphological divergence of their hosts in sympatric ecomorphs; by addressing the hypothesis using these two parallel evolutionary scenarios of their hosts, we acknowledge the existing of gene flow among host populations; we try to uncover the forces that may shape the genetic structure of the parasite and determine if parasite divergence is mediated by host ecotype, host speciation or other aspects related to the environment or the intermediate hosts involved in the life cycle. This study was then designed to use a phylogenetic analysis among populations of *P.* (*S.*) *neocaballeroi* as a framework to answer the following questions: (a) Does *P.* (*S.*) *neocaballeroi* show a genetic structure associated with the ecological differences of their hosts (cavefish and surface fish)? (b) Does *P.* (*S.*) *neocaballeroi* show a genetic structure associated with host niche specialization in sympatric hosts in a lacustrine system (*A. caballeroi* and *A. aeneus*)? (c) What are the forces that determine the uncovered pattern of genetic structure of this highly specific species of nematode?

2 | MATERIAL AND METHODS

2.1 | Sample collection

Individuals of *Astyanax* (*A. aeneus*, *A. caballeroi* and *A. mexicanus*) were sampled between 2015 and 2019 from 52 localities in different river basins in Mexico and Central America, including the type locality of *P.* (*S.*) *neocaballeroi*,

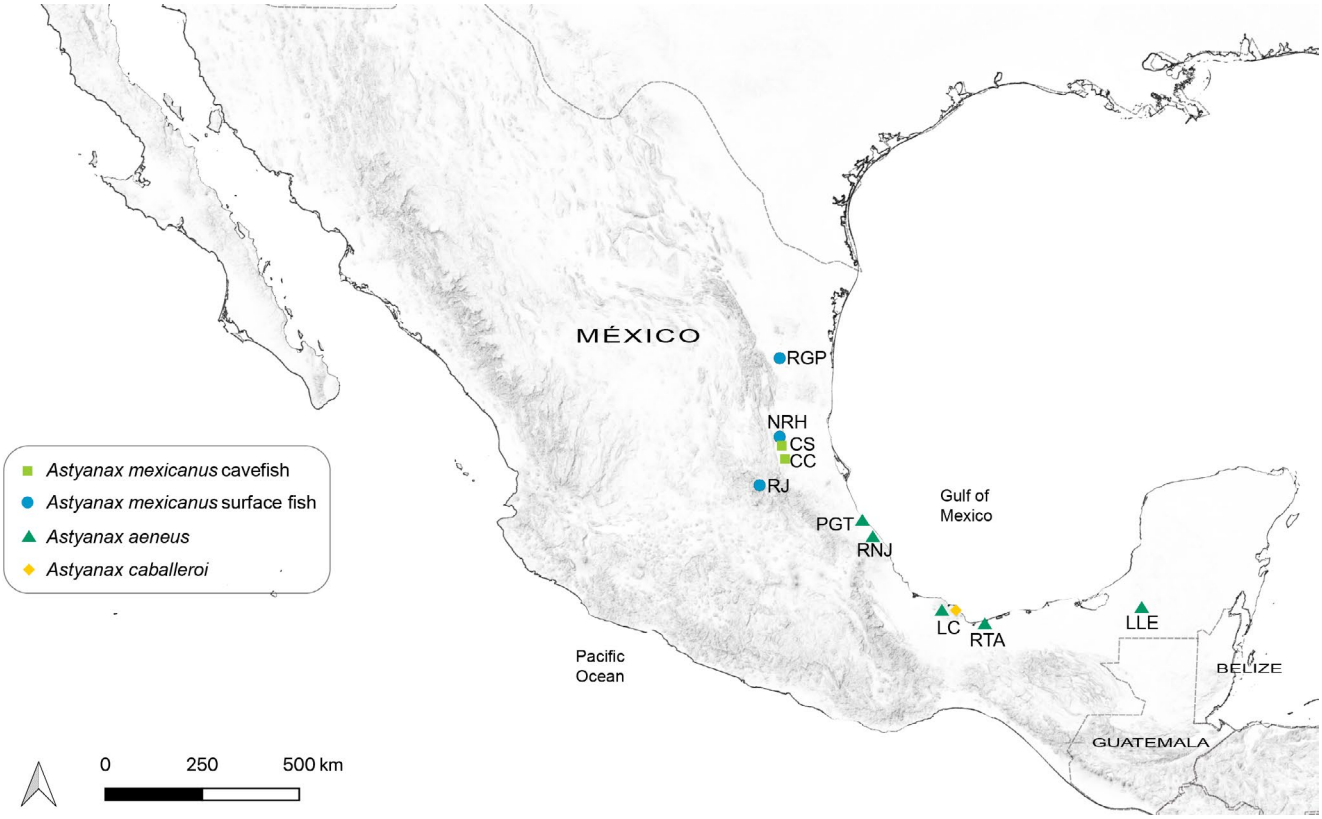

FIGURE 1 Map showing the 10 localities (out of the 52 sampled) were some species of *Astyanax* resulted positive to the infection by *P.* (*S.*) *neocaballeroi*. Each colour and shape symbol represent each *Astyanax* species. The letter codes indicate the sampling locality according to Table S1

Lake Catemaco, Veracruz, Mexico. Eight of these localities corresponded to cavefish populations of *A. mexicanus* from the Sierra de El Abra, in northeastern Mexico, (Figure 1, Table S1). Fishes were euthanized with tricaine (MS-222) and dissected for parasitological analysis. Nematodes were recovered from the intestine and/or stomach of their hosts and immediately fixed in 100% EtOH for molecular and morphological analyses. The parasite prevalence, abundance and mean intensity of nematodes for each combination of host species and sampling site were calculated following Rózsa et al. (2000).

2.2 | Molecular methods

Genomic DNA was isolated from a fragment of a single nematode (hologenophore sensu Pleijel et al., 2008), using DNAzol reagent (Thermo Fisher Scientific) following the fabricant instructions. DNA was extracted from 40 specimens of *P.* (*S.*) *neocaballeroi* from 10 populations: La Libertad, Campeche (7); Río Guayalejo Tamaulipas (1); Río Jalpan, Querétaro (2); Cueva Chica (5), Cueva Sabinos (7) and Río Huichihuayán (1), San Luis Potosí; Lago Catemaco, (7), Río Tecolutla (7), Río Nautla (2), and Río Tonalá (1), Veracruz (Figure 1). All PCRs were conducted in 25 µl reactions containing 0.5 µl of My Taq (Bioline), 1 µl of each primer 10 mM, 2.5 µl PCR buffer, 14.375 µl of nuclease-free water and 2 µl of DNA template. The primers used for COI and 28S rDNA are listed in Table S2. The following thermocycler setting for PCR was performed: initial denaturation at 94° for 2 min, followed by 30 cycles at 94°C for 1 min, annealing temperature of 48°C for COI and 50°C for 28S rDNA for 1 min, 72°C for 2 min and final extension at 72°C for 7 min. PCR products were purified using Exo-SAP-IT (Affymetrix) and Sanger sequencing reaction with Big Dye (Applied Biosystems). The sequences were obtained in the Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud, UNAM or in Macrogen Inc., Seoul, South Korea.

2.3 | Sequence alignment and phylogenetic analyses

DNA sequences were assembled using Geneious v7 (Kearse et al., 2012) and aligned with Clustal Omega implemented in the Analysis Tool Web Services from the EMBL-EBI (McWilliam et al., 2013). All the obtained sequences were submitted to GenBank with accession numbers MT579478–MT579519 for COI and MT582720–MT582743 for the

28S rDNA (Table S3). The newly sequenced individuals were used along with sequences of specimens available in GenBank, for the COI alignment, sequences of *Camallanus cotti* (EU598890), *C. hypophthalmichthys* (EU598816), *P. spiculogubernaculus* (KU292358), *Spirocamallanus huacraensis* (MK780067), *S. istiblenni* (KC517405) and new sequences of *P.* (*S.*) *gobiomori*. In the phylogenetic analyses, the sequence of *Dracunculus lutrae* (EU646614) was used as outgroup following phylogenetic analyses of Choudhury & Nadler (2016). For the 28S rDNA alignment and the concatenated data sets, the sequence of *S. huacraensis* (MK793794) was included as outgroup.

The best fit model for each matrix was calculated at ATGC bioinformatics platform using the Smart Model Selection (SMS) (Lefort, Longueville & Gascuel, 2017), according to the Akaike information criterion (AIC). The phylogenies for each gene and the concatenated matrix (COI + 28S) were constructed with maximum likelihood (ML) using the online software PhyML 3.1 (Guindon et al., 2010), performed with 10,000 bootstrap replicates under the GTR + G substitution model. In addition, Bayesian inference (BI) was implemented in MrBayes (Huelsenbeck & Ronquist, 2001), using the GTR + G model of nucleotide substitution. To perform the BI, CIPRES Science Gateway Web Portal V3.3 was used (Miller et al., 2010). The analyses consisted of two runs of four chains, each for 10 million generations, sampling trees every 1,000 generations and a temperature of 0.25. The first 20% of trees were discarded as burn-in, and a 50% majority-rule consensus tree was constructed from the postburn-in trees. Tracer v.1.7 (Rambaut et al., 2018) was used to assess convergence and adequate posterior sampling (ESS > 200). All the trees generated were visualized in FigTree v1.4.3 (Rambaut, 2016).

2.4 | Genetic diversity and species delimitation

The aligned DNA sequences were analysed in DNAsp v6 (Rozas et al., 2017) to calculate statistics for COI gene: number of haplotypes, haplotypic diversity and nucleotide diversity. A haplotype network was created with the minimum spanning network in PopART (Leigh & Bryant, 2015) using the ML tree as input. The uncorrected *p-distances* were calculated in MEGA v.7 (Kumar, Stecher & Koichiro, 2015) with variance estimation with bootstrap method using 10,000 replications. To examine differentiation among the ten parasite populations in the predefined lineages, pairwise F_{ST} values based on pairwise genetic differences were calculated using Arlequin v3.5 (Excoffier & Lischer, 2010). In addition, an analysis of molecular variance (AMOVA) using pairwise differences was conducted to further test the validity of the lineages suggested by the phylogenetic reconstruction. The significance of the variance components was assessed with the permutation tests (10,000 replicates) in Arlequin.

For the species delimitation, a tree-based approach was used, in an analysis of multi-rate Poisson tree processes (mPTP) (Kapli et al., 2017). As input file was used, the rooted tree of the phylogenetic inference was conducted with ML using the mitochondrial sequences. We used a Markov Chain Monte Carlo (MCMC) sampling method for assessing the confidence of the maximum likelihood delimitation scheme, and we performed two independent MCMC runs of 10^8 generations, sampling every 1,000 iterations, each run started with a random delimitation approach. The convergence was assessed with the visual inspection of the combined likelihood plot of both runs.

2.5 | Morphological analyses

For the morphological study of nematodes, hologenophores of the uncovered genetic lineages were cleared in a glycerol-alcohol solution (1:1) to observe and measure some taxonomically important features; an Olympus BX51 light inverted microscope equipped with differential interference contrast (DIC) optical components was used. To study the external surface of body, specimens were prepared for scanning electron microscopy (SEM); each individual was sonicated, dried to critical point, mounted on a strip of carbon conductive tape and coated with gold. The SEM photographs were taken in a Hitachi scanning electron microscope 15 kV Hitachi Stereoscan Model SU1510.

3 | RESULTS

3.1 | Phylogenetic relationships

We generated 40 COI sequences of *P.* (*S.*) *neocaballeroi* of *Astyanax* spp. from 10 populations and additional sequences of *P.* (*S.*) *gobiomori* from eleotrid hosts. The alignment was 802 bp long. The ML and BI approaches yielded similar topologies, both showing clusters separated by host species and geographical distribution. All COI sequences of *P.* (*S.*) *neocaballeroi* were monophyletic; within this group, we found three lineages with high nodal support for Lineages 1 and 2, and low support for Lineage 3 (Figure 2). The first lineage (L1) included populations from the Mexican tetra, *A. mexicanus* in northeastern Mexico, including two caves, Sabinos and Chica (CS and CC), as well as surface populations from Tamaulipas, Querétaro and San Luis Potosí (NRH, RJ, RGP) in the Panuco and Soto La Marina river basins. A second lineage (L2) contained specimens from the Banded tetra, *A. aeneus* from Campeche

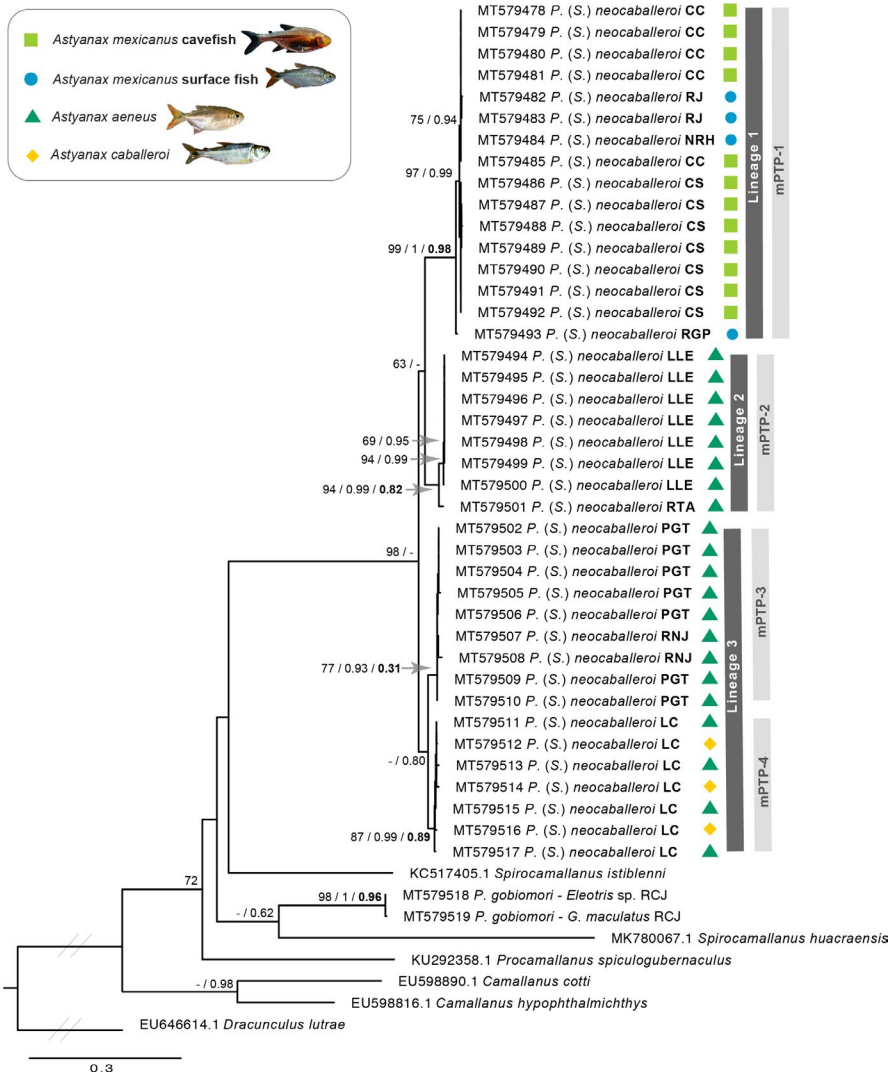

FIGURE 2 COI phylogenetic tree of *P.* (*S.*) *neocaballeroi*. Phylogenetic tree obtained by maximum likelihood (ML); bootstrap and posterior probability support values above 60/0.6, respectively, is shown in the internodes. The support values for the speciation events yielded with the mPTP analyses are shown in the corresponding nodes after the bootstrap and posterior probability. Each colour and shape symbol represent each *Astyanax* species. The light grey bars show the results of the mPTP analyses, and the letter codes indicate the sampling locality as in Table S1

and southern Veracruz (RTA and LLE) in the Grijalva-Usumacinta river basins. Finally, a third lineage (L3) included the nominal *P.* (*S.*) *neocaballeroi* from the type locality (Lake Catemaco, Veracruz, LC) parasitizing two sympatric species of *Astyanax*, that is *A. aeneus* and *A. caballeroi*, and two additional populations (PGT and RNJ) in the Papaloapan river basin (Table S1). The 28S rRNA gene was sequenced for 24 nematodes, representing a subset of each COI lineage of *P.* (*S.*) *neocaballeroi*. Sequences were 977–1,143 bp long, with 19 variable sites. The phylogenetic reconstruction based on the 28S rDNA yielded low resolution (tree not shown), showing that this nuclear fragment is conserved. For the concatenated data set (COI + 28S rDNA), the phylogenetic tree reconstruction yielded the same three lineages of *P.* (*S.*) *neocaballeroi* obtained with the mitochondrial data; however, Lineage 3 is recovered with moderate nodal support (Figure S1). Lineages are not geographically overlapped and display heterogeneous patterns of infection, for example high prevalence in cave environments (Table S4, Figure S2).

3.2 | Genetic diversity, haplotype network and species delimitation

The genetic divergence (*p-distance*) among isolates of each of the lineages of *P.* (*S.*) *neocaballeroi* ranged between 0.40% and 1.61% (highest in *P.* (*S.*) *neocaballeroi* L3). Among the three lineages of *P.* (*S.*) *neocaballeroi*, genetic divergence varied from 5.12% to 6.91%. Instead, the genetic divergence between lineages of *P.* (*S.*) *neocaballeroi* and *P.* (*S.*) *gobiomori* varied from 14.33% to 21.04%, and the observed differences between lineages of *P.* (*S.*) *neocaballeroi* and other members of the family Camallanidae ranged between 16.45% and 21.04% (Table 1). The nucleotide and haplotype diversity were relatively high within each lineage of *P.* (*S.*) *neocaballeroi;* in total, 21 haplotypes were identified (Table 2). The haplotype network analysis of the mitochondrial haplotypes agreed with the phylogenetic analysis and also yielded three clusters separated by numerous mutations, and no haplotypes were shared among lineages (Figure 3). The haplotypes corresponding to

TABLE 1 COI genetic distances among lineages of *Procamallanus* (*Spirocamallanus*) *neocaballeroi* and between *Procamallanus* (*Spirocamallanus*) *neocaballeroi* and other species of camallanids. Intraspecific genetic distance is in parenthesis. Genetic distances are below the diagonal and standard error is above the diagonal. Values are represented as percentages

	Lineage	Distance within lineage (*SE*)	Distance between lineages									
			1	2	3	4	5	6	7	8	9	10
1	*P.* (*S.*) *neocaballeroi* L1	0.46 (0.15)		0.84	0.86	1.51	1.99	1.65	1.83	1.98	2.14	1.69
2	*P.* (*S.*) *neocaballeroi* L2	0.40 (0.12)	6.44		0.70	1.49	1.94	1.53	1.78	1.90	2.09	1.65
3	*P.* (*S.*) *neocaballeroi* L3	1.61 (0.32)	6.91	5.12		1.48	1.87	1.52	1.78	1.91	2.04	1.62
4	*P.* (*S.*) *gobiomori*	0.17 (0.16)	16.53	15.66	16.10		1.90	1.47	1.71	1.74	1.85	1.63
5	*S. istiblenni*	—	16.45	16.26	16.18	14.50		1.91	1.90	1.90	1.97	2.07
6	*P. spiculogubernaculus*	—	17.33	15.35	14.48	14.33	17.25		1.85	1.89	1.89	1.66
7	*C. cotti*	—	19.23	17.26	17.76	15.30	17.13	18.73		1.62	2.04	1.91
8	*C. hypophthalmichthys*	—	20.24	18.94	19.17	15.34	15.51	18.53	12.65		2.06	1.93
9	*S. huacraensis*	—	21.04	20.93	20.33	16.15	17.18	19.52	21.19	20.21		2.14
10	*D. lutrae*	—	24.28	22.26	23.10	23.82	24.59	22.68	23.36	24.12	28.87	

Abbreviation: SE, standard error.

P. (*S.*) *neocaballeroi* Lineage 1 exhibit at least one shared haplotype between *A. mexicanus* from Chica Cave (CC) and the surface populations of Huichihuayán river (NHR). In *P.* (*S.*) *neocaballeroi* Lineage 2, three private haplotypes were uncovered, two of them connected by several mutations to one haplotype of Lineage 3, suggesting a case of reticulate evolutionary relationship among haplotypes.

In Lineage 1, the pairwise F_{ST} shows a significant amount of differentiation between Cave Sabinos and Cave Chica populations ($F_{ST} = 0.8889$, $p = .001$). In Lineage 2, the number of sequences per population limits the comparisons, and in Lineage 3, all the pairwise comparisons displayed significative differentiation. However, between the nematodes infecting sympatric *Astyanax* in the lacustrine system, there is no genetic subdivision. The AMOVA analysis indicates that most of the variation occurs among lineages (79.76% $p < .001$), which suggests strong genetic divergence (Tables S5 and S6).

Furthermore, the species delimitation analyses conducted through mPTP partitioned *P.* (*S.*) *neocaballeroi* in a species complex containing four candidate species; *P.* (*S.*) *neocaballeroi* Lineages 1 and 2 were yielded as independent evolutionary units, with high nodal support values. However, *P.* (*S.*) *neocaballeroi* Lineage 3 which corresponded to *P.* (*S.*) *neocaballeroi* sensu stricto, since it was collected from the type locality, was partitioned into two additional species, although only one of the candidate species reach high support in the mPTP analyses (Figure 2).

3.3 | Morphological data

Overall, morphology of the sampled specimens of nematodes correspond with the original description of *P.* (*S.*) *neocaballeroi* (Caballero- Deloya, 1977). The species was originally described as a parasite of *Astyanas fasciatus* (= *Astyanax aeneus*) in Lake Catemaco, Veracruz. The specimens can be readily distinguished from other species of *Procamallanus* spp. infecting non-characid host, even with those occurring in sympatry. However, morphological traits do not allow us, at the moment, to establish a reliable distinction among specimens from the three genetic lineages. Moreover, measurements of the main diagnostic traits overlapped. In this study, we also obtained scanning electron microphotographs of specimens from the three genetic lineages looking for ultrastructural

TABLE 2 Genetic diversity of COI sequences from *Procamallanus* (*Spirocamallanus*) *neocaballeroi* species complex

Lineage	Localities	ns	nh	∏ (SD)	hd (SD)	k
P. (*S.*) *neocaballeroi* Lineage 1	CS, CC, NRH, RGP, RJ	16	7	0.00458 (0.00083)	0.817 (0.073)	3.66667
P. (*S.*) *neocaballeroi* Lineage 2	LLE, RTA	8	3	0.00397 (0.00261)	0.464 (0.200)	3.17857
P. (*S.*) *neocaballeroi* Lineage 3	LC, PGT, RNJ	16	11	0.01649 (0.00157)	0.933 (0.048)	12.00833

Abbreviations: ∏, nucleotide diversity (SD, standard deviation); k, mean number of nucleotide differences; nh, number of haplotypes; ns, number of sequences.

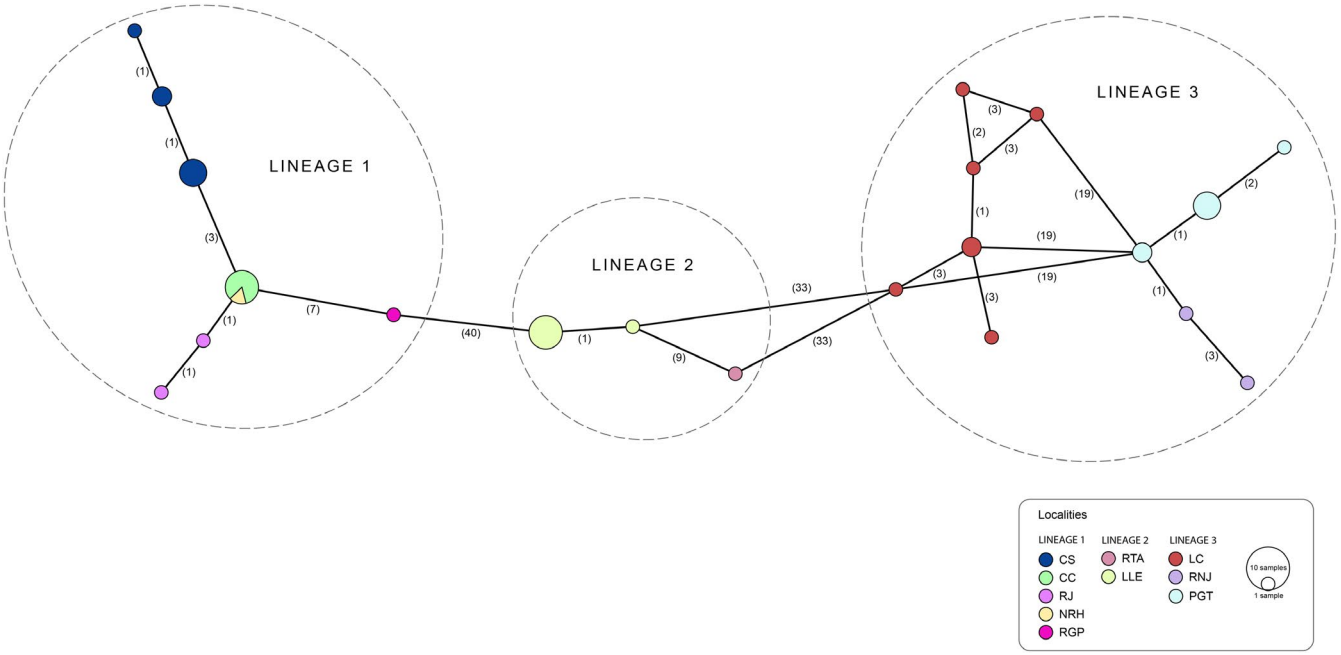

FIGURE 3 Haplotype network based on the COI matrix of *P.* (*S.*) *neocaballeroi*. The size of the circle indicates the number of individuals sharing the same haplotype. The colour of the circle is in reference to the sampling locality. Number of mutational steps among haplotypes are in parenthesis

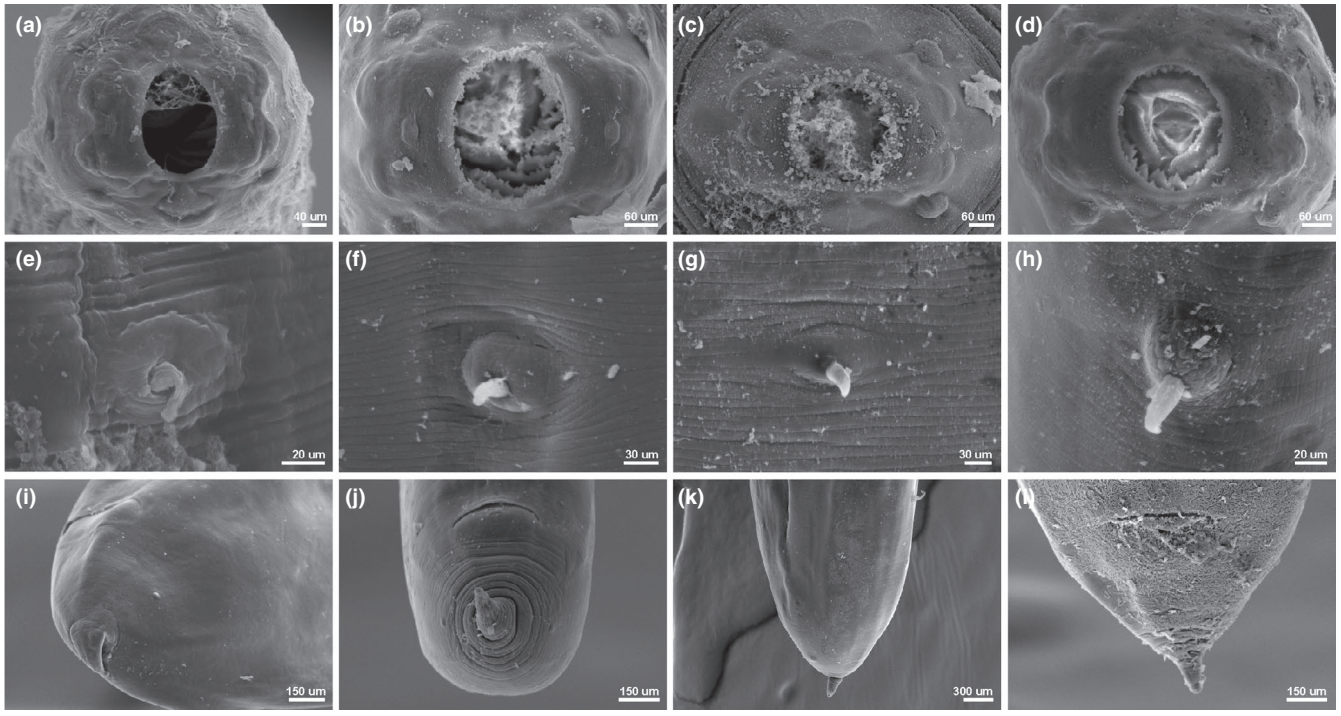

FIGURE 4 Scanning electron microscopy microphotographs of female specimens of *P.* (*S.*) *neocaballeroi*. Lineage 1 (a, e, i); Lineage 2 (b, f, j); Lineage 3 ex *Astyanax aeneus* (c, g, k), Lineage 3 ex *A. caballeroi* (d, h, i). Upper row shows comparative apical views of the cephalic end; middle row shows small and simple deirids; lower row shows rounded tail with a small, rounded digit-like protrusion, lacking terminal spike

characters that may assist in the species differentiation (Figure 4). We did not observe differences among the three lineages. Unfortunately, some of the sampled individuals corresponded to immature forms with incomplete development of key morphological characters. Within the genus *Procamallanus (Spirocamallanus)*, characters such as the oral capsule shape, the shape of the female cauda and the papillae arrangement in the male tail are taxonomically very important characters to discriminate species. Figure 4 shows a comparison of these morphological traits among

the genetic lineages of *P*. (*S*.) *neocaballeroi* although no conspicuous ultrastructural differences can be observed; specimens exhibit a very similar shape of the female cephalic end (in apical view) (Figure 4a–d), with four prominent submedian cephalic papillae and one pair of amphids; all females possess small and simple deirids (Figure 4e–h); and all the females exhibit the characteristic rounded tail with a small, rounded digit-like protrusion, lacking terminal spike (Figure 4i–l).

4 | DISCUSSION

In this study, we evaluated the genetic diversity of the nematode *P*. (*S*.) *neocaballeroi* occurring in different ecotypes of the freshwater fish *Astyanax* spp. across a wide geographical range. The results of our analyses using either the mitochondrial gene COI, or the concatenated analysis of the COI + 28S genes, yielded no genetic differentiation associated with host habits. Instead, genetic lineages of the parasite were recovered according to host species distribution and geographic region. *Procamallanus* (*S*.) *neocaballeroi* Lineage 1 is restricted to the Mexican tetra, *A. mexicanus* including both, surface and cave-dwelling populations; *P*. (*S*.) *neocaballeroi* Lineage 2 occurs in the Banded tetra, *A. aeneus* and *P*. (*S*.) *neocaballeroi* Lineage 3 was found in two species of *Astyanax, A. caballeroi* and *A. aeneus*, in congruence with the geographic distribution pattern of each host species. By using one mitochondrial and three nuclear DNA genes to evaluate the phylogenetic relationships within the Middle American species of *Astyanax*, Ornelas-García et al. (2008) uncovered that populations from North America and upper Central America formed a monophyletic group, whereas those from Middle America experienced a rapid radiation with unresolved relationships. Still, Ornelas-García et al. (2008) found two genetic lineages for *A. mexicanus* and three for *A. aeneus*, and these lineages correspond largely with the lineages of *P*. (*S*.) *neocaballeroi* we found in our study. In contrast, the genetic structure of the nematode populations exclusively found *in A. mexicanus* do not fully reflect the reticulate evolution of *A. mexicanus*, involving multiple and independent invasions of the cave and surface environments (see Gross, 2012), or even subterranean connections between caves (Herman et al., 2018).

A similar pattern was shown to occur in the spirurid nematode *Rhabdochona* spp., also in association with *Astyanax* spp. (Santacruz et al., 2020). Given the high levels of host specificity uncovered in our study, we might speculate that a speciation event of the host resulted in a barrier for the parasite and generated reproductive isolation (Avise, 2004). Thus, allopatric diversification as a result of the complex hydrological history of river basins produced isolation of fish and parasite populations. However, we cannot discern at the moment whether the observed divergence is the product of geographic isolation, the specific interaction with its host or both.

The genetic divergence values obtained among the lineages of *P*. (*S*.) *neocaballeroi* were high and correspond with those observed among different species of nematodes (e.g. Solórzano-García, Nadler & Pérez-Ponce de León, 2016; Mockett et al., 2017; Lagunas-Calvo et al., 2019). In many cases, relatively low levels of genetic divergence are accompanied by the absence of conspicuous morphological changes, leading to the recognition of cryptic species complexes (see Pérez-Ponce de León & Nadler, 2010; Nadler & Pérez-Ponce de León, 2011; Pérez-Ponce de León & Poulin, 2018). Our results indicate an incipient genetic divergence of *P*. (*S*.) *neocaballeroi*, accompanied by the lack of conspicuous of morphological differences among the three lineages (Figure 4); detailed observations of the specimens through light and scanning electron microscopy yielded no evident morphological differences among the studied specimens; we acknowledge, however, that we could not detect such differences at this time because some specimens were found as immature adults, and our sampling size was relatively small given the low prevalence and abundance of the nematode (Table S4). Likewise, other sources of evidence, such as reciprocal monophyly for each lineage in the phylogenetic reconstruction (Figure 2; Figure S1) albeit with low nodal support for Lineage 3, a strong genetic structure of *P*. (*S*.) *neocaballeroi* among host populations (Figure 3), host specificity and non-overlapping geographic distribution allowed us to conclude that *P*. (*S*.) *neocaballeroi* represents a cryptic species complex.

Taxonomically, one of the lineages (i.e. *P*. (*S*.) *neocaballeroi* Lineage 3) represents the species *P*. (*S*.) *neocaballeroi* sensu stricto since it corresponds with the type locality, Lake Catemaco, Veracruz (Caballero- Deloya, 1977); the other two lineages, (*P*. (*S*.) *neocaballeroi* Lineage 1 and Lineage 2), may represent independent evolutionary units following the species concept of de Queiroz (2007). The species delimitation analyses actually yielded four independent evolutionary units instead of three (Figure 2), with the population of *P*. (*S*.) *neocaballeroi* from Lake Catemaco in Veracruz (LC) representing two separate species of the rest of the species in the complex. As discussed above, we did not find reliable morphological differences among the specimens we sampled during the course of this investigation; we were unable to describe (and name) two or three evolutionary units uncovered in our study. This part of the investigation requires further scrutiny in order to sample more specimens from these localities and conduct an even more detailed morphological study in the search for distinguishing characters. Also, such study would contribute to generate additional empirical data to

resolve the problematic related to the subgeneric status of the species included in the genus *Procamallanus*. Several authors have provided molecular data demonstrating that the subdivision of the genus into several subgenera based on the structure of the buccal capsule should be abandoned (Černotíková et al., 2011; Sardella et al. 2017; Ailán-Choke et al., 2019). We took a conservative position and decided to still use the traditional classification, as *Procamallanus* (*Spirocamallanus*) *neocaballeroi*.

The analyses of our study also showed that cave and surface nematode populations parasitizing *A. mexicanus* are not isolated. Several evidences may explain the genetic admixture between populations. Even though the Mexican tetra occurs in contrasting habitats, each host represents a constant environment for the parasite, acting as a buffer/balancing selection force of the contrasting environment where the host lives. Furthermore, it has been shown that some caves display introgression of surface fishes to the caves (Bradic et al., 2012), and even interbreed with the cavefish; this may have been promoting the parasite interchange. An alternative possibility is the movement of the intermediate host in and out of the caves (actively or during flooding events). Juvenile cavefish are predators of aquatic crustaceans (Espinasa et al., 2017), which are the regular intermediate hosts in the life cycle of *P.* (*S.*) *neocaballeroi* (Moravec & Vargas-Vázquez, 1996). Larval forms of the nematode are acquired by the fish by consuming crustaceans, although a change in diet occurs when fish grow and become adults (Espinasa et al., 2017). The fact that individuals of *A. mexicanus* from caves or surface habitats might be infected with the nematode *P.* (*S.*) *neocaballeroi* supports the recent findings of Ornelas-García et al. (2018); these authors demonstrated that the contrasting environment between cave and surface does not account for differences of the microbiome of fish populations. Still, more studies are required to fully understand the relationships between fish and nematodes in these contrasting environments. It is necessary to explore the causes of the higher prevalence of infection by *P.* (*S.*) *neocaballeroi* in fishes living in caves than those in the surface. Moreover, we have observed in some of the cave populations studied thus far that the helminth parasite species richness is lower in caves, in comparison with surface-dwelling fishes (Santacruz, 2013). Temperature preference in cavefish which contrast to the surface fish (Tabin et al., 2018) could account for some of these difference of prevalence of infection by *P.* (*S.*) *neocaballeroi* in both environments, since temperature affect host–parasite interaction, as shown by Franke et al. (2017) studying sticklebacks infected with the tapeworm *Schistocephalus solidus*.

Lastly, our study revealed no genetic differentiation among populations of *P.* (*S.*) *neocaballeroi* occurring in the intestine of *A. caballeroi* and *A. aeneus* in the type locality (Lake Catemaco). These two fish species occur in sympatry and show clear patterns of ecological disparity (Ornelas-García et al., 2014; Ornelas-García et al., 2018). It might be possible that the two species of lake-dwelling fishes could share the same intermediate host of the parasite, since both morphotypes partially share a trophic niche (Ornelas-García et al., 2018). More information is still required to fully understand the role that hosts may have on the evolutionary history of the parasites and the fact that they may play a major role on their diversification history. Whether or not patterns are congruent will rely on several forces that shape the evolutionary and biogeographical history of both associates.

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CONFLICT OF INTEREST

None.

DATA AVAILABILITY STATEMENT

New sequence data were deposited in the GenBank. Accession numbers of each isolate are included in the phylogenetic trees and the Table S3.

ORCID

Ana Santacruz https://orcid.org/0000-0003-2673-1322
Claudia Patricia Ornelas-García https://orcid.org/0000-0003-3163-448X
Gerardo Pérez-Ponce de León https://orcid.org/0000-0001-6472-5113

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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