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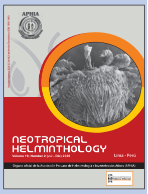

ORIGINAL ARTICLE / ARTÍCULO ORIGINAL

NEOBENEDENIA CIBNORENSIS N. SP. (MONOGENEA: CAPSALIDAE), A CRYPTIC SPECIES INFESTING THE SKIN OF *SERIOLA RIVOLIANA* VALENCIENNES, 1833 (PERCIFORMES: CARANGIDAE) FROM LA ENSENADA DE LA PAZ, BAJA CALIFORNIA SUR, MEXICO

NEOBENEDENIA CIBNORENSIS N. SP. (MONOGENEA: CAPSALIDAE) UNA ESPECIE CRÍPTICA QUE INFESTA LA PIEL DE *SERIOLA RIVOLIANA* VALENCIENNES, 1833 (PERCIFORMES: CARANGIDAE) EN LA ENSENADA DE LA PAZ, BAJA CALIFORNIA SUR, MÉXICO

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ABSTRACT

Ectoparasites of the genus *Neobenedenia* Yamaguti, 1963, are responsible for causing mortality within aquaculture systems. Given their pronounced morphological resemblance, the identification of *Neobenedenia* species based on phenotypic characteristics poses significant challenges. Thus, molecular techniques are used to complement discrimination between species with very limited morphological variation, such as *Neobenedenia melleni* (MacCallum, 1927) Yamaguti 1963 and

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N. girellae (Hargis, 1955) Yamaguti, 1963. Molecular markers can enhance the ability to describe biological diversity and understand the biogeography of these species. The gulf of California has traditionally hindered the exchange of genes for various species with different life histories. In this study, *Neobenedenia* specimens were collected from wild and captive *Seriola rivoliana* Valenciennes, 1833, at four locations within the Gulf of California. Partial sequences of the 28S rDNA and Cytochrome b (Cytb) genes revealed genetic divergence among the specimens. Cytb analysis showed that specimens from cultured *S. rivoliana* at La Ensenada de La Paz are genetically distinct from *N. girellae* found in other Pacific Ocean localities. Principal Component Analysis (PCA) also revealed morphometric differences, supporting the recognition of these specimens as a new species, *Neobenedenia cibnorensis* n. sp. These findings highlight the presence of at least two distinct *Neobenedenia* species infecting wild and captive *S. rivoliana* populations within the gulf of California. The integration of molecular and morphometric analyses was key to the accurate identification of these cryptic species and has implications for parasite management and biosecurity in *Seriola* Cuvier 1816, aquaculture in the region.

Keywords: cryptic species - morphometric analysis - *Neobenedenia cibnorensis* n. sp. – phylogeny - *Seriola rivoliana* - species delimitation

RESUMEN

Las especies del género *Neobenedenia* Yamaguti, 1963, son ectoparásitos responsables de causar mortalidad en sistemas de acuicultura. Debido a su marcada similitud morfológica, la identificación taxonómica basada únicamente en características fenotípicas presenta importantes desafíos. Por ello, las técnicas moleculares se han convertido en herramientas clave para distinguir entre especies morfológicamente similares, como *Neobenedenia melleni* (MacCallum, 1927) Yamaguti 1963 y *N. girellae* (Hargis, 1955) Yamaguti, 1963. El uso de marcadores moleculares permite mejorar la descripción de la diversidad biológica y comprender la biogeografía de estos parásitos. El Golfo de California ha actuado históricamente como una barrera al flujo génico para diversas especies marinas con distintos tipos de historia de vida. En este estudio, se recolectaron ejemplares de *Neobenedenia* en individuos silvestres y en cultivo de *Seriola rivoliana* Valenciennes, 1833, en cuatro localidades del golfo de California. El análisis de secuencias parciales de los genes 28S rDNA y citocromo b (Cytb) reveló divergencia genética entre los ejemplares. El análisis del gen Cytb indicó que los especímenes provenientes de peces cultivados en La Ensenada de La Paz son genéticamente distintos de *N. girellae* registrada en otras localidades del océano Pacífico. Además, los resultados del Análisis de Componentes Principales (PCA) mostraron diferencias morfométricas, lo que respalda su designación como una nueva especie: *Neobenedenia cibnorensis* n. sp. Nuestros hallazgos evidencian la presencia de al menos dos especies distintas de *Neobenedenia* que infestan a *S. rivoliana*, tanto en poblaciones silvestres como en cultivo dentro del golfo de California. La combinación de herramientas moleculares y análisis morfométricos fue crucial para la identificación precisa de esta especie críptica y resulta relevante para el manejo de parásitos en sistemas de cultivo de *Seriola* Cuvier 1816, en la región.

Palabras clave: análisis morfométrico - delimitación de especies - especies crípticas – filogenia - *Neobenedenia cibnorensis* n. sp. - *Seriola rivoliana*

INTRODUCTION

Neobenedenia Yamaguti, 1963, a skin monogenean parasite, significantly impacts aquaculture by causing diseases, reducing production, and increasing mortality rates (Whittington, 2012). This parasite's rapid multiplication, facilitated by its direct life cycle, poses a considerable threat to profitability and viability (Hirazawa *et al.,* 2016). In *Seriola* Cuvier 1816, aquaculture, capsalids contribute to substantial economic losses, which can amount to as much as a quarter of the total production cost (Sepúlveda & González, 2019 and reference therein).

Seriola rivoliana Valenciennes, 1833 (commonly known as Almaco jack) has increased in commercial importance, and various countries, including Mexico, have implemented strategies for its cultivation (Roo *et al.*, 2014; Sicuro & Luzzana, 2016). Genetic differences have been described in *S. rivoliana* populations inside and outside the Baja California Peninsula within the Gulf of California, due to barriers in gene flow produced by oceanographic structures such as eddies and fronts (Mendoza-Portillo *et al.*, 2020).

The Gulf of California and adjacent Pacific Ocean display phylogeographic discontinuities among diverse species, documented primarily through mitochondrial DNA analysis. These findings suggest that the Gulf of California entrance acts as a gene flow barrier for species with varied life histories (Bernardi *et al.,* 2003; García-De León *et al.*, 2018). Mesoscale oceanographic processes in the area are hypothesized to hinder the dispersal of early fish life stages (Saavedra-Soleto *et al.*, 2013; Sánchez-Velasco *et al.*, 2025).

Recent molecular data confirm distinctions between *N. girellae* (Hargis, 1955) Yamaguti, 1963, and *N. melleni* (MacCallum, 1927) Yamaguti 1963, resolving a long-standing debate (Brazenor *et al.,* 2018). These globally distributed species, unlike most monogeneans, infect multiple teleost hosts and are considered cryptic species due to morphological similarity (Brazenor *et al.,* 2018 and reference therein). However, Sepúlveda & González (2019) identified a genetically distinct species on the northern coast of Chile that infects cultured *Seriola lalandi* Valenciennes, 1833, in a hatchery, different from *N. girellae* or *N. melleni*.

Incorrectly defining parasitic species boundaries can complicate research on local parasite adaptation, host specificity, and disease dynamics (Sepúlveda & González, 2019 as well as the citations within). For this reason, identifying cryptic species has become increasingly vital in taxonomy and biodiversity research, and the use of DNA sequencing has greatly aided in the differentiation of species with similar morphological characters (Bickford *et al.,* 2007; Detwiler *et al.,* 2010; Padial *et al.,* 2010; Halnet *et al.,* 2015; Struck *et al.,* 2018).

Therefore, the aim of this study was to analyze the genetic variation using two molecular markers, 28S rDNA and Cytochrome b (Cytb) of *Neobenedenia* sp. specimens from wild and farmed *S. rivoliana* in Baja California Sur within the Gulf of California, Mexico. The information will contribute to a better understanding of the ecology of this taxon, which is implicated in damage to wild and farmed aquaculture fish.

MATERIAL AND METHODS

Sample collection

Neobenedenia specimens were obtained from wild and captive *S. rivoliana* between 2014 and 2019 at four locations in the East and West Coast of Baja California Sur, Mexican Pacific (Fig. 1). Live parasites were retrieved directly from the host skin or recovered through freshwater fish immersion, which terminated the parasites. The specimens collected were then preserved in 96% ethanol.

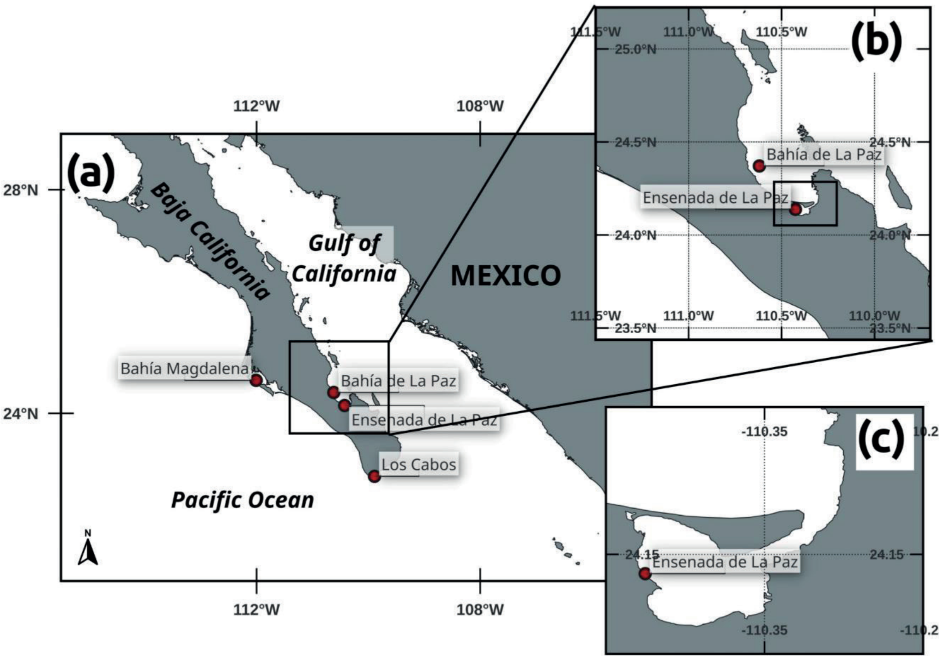

Figure 1. Collection sites of *Neobenedenia* spp. infesting *Seriola rivoliana* in the South of Baja California, Mexico (a) *Neobenedenia cibnorensis* n sp. and *Neobenedenia girellae* from Bahía de La Paz and Ensenada de La Paz (b) *Neobenedenia cibnorensis* n. sp. from Ensenada de La Paz.

Morphological data

Monogeneans were dehydrated with different concentrations of alcohol to be stained with Gomori´s trichomic, cleared with eugenol and mounted in Canada balsam. Measurements were made using a Carl Zeiss microscope (Carl Zeiss AG, Germany). Measurements are given in micrometers (µm), as mean values followed by the standard deviation and, range (minimum-maximum). The parasites were deposited in the Colección de Parásitos de Peces del Noroeste del Pacífico (CPPNP) from Centro de Investigación en Alimentación y Desarrollo, Unidad Mazatlán, Mexico.

Morphometric analyses

Morphometric measurements from all 359 *Neobenedenia* specimens, from both literature records and from this study (see supplementary Table S1), were analyzed using Principal Component Analysis (PCA). In the PCA the following morphometric measurements were considered: (1) mean body length (length_mean), (2) mean body width (width_mean). In addition, we calculated somebody proportions that were also included in the PCA: (3) body width to body length ratio, (4) the hamulus length to body length ratio (hl_ratio), and (5) the sclerites length to the body length ratio (sl_ratio). A single classification ANOVA was used to test differences in parasite body length among host species, at a 95% confidence level. All analyses were performed with R studio 4.1.2 (R Core Team, 2021).

Nucleic acid extraction, PCR amplification and sequencing

Genomic DNA was extracted based on the modified cetyl-trimethylaminium bromide (CTAB) buffer protocol described in Valles-Vega *et al.* (2019). For each DNA extraction, two ectoparasites were placed in a 2 mL Eppendorf tube containing 250 µl of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) and left at room temperature for one hour, after which the buffer was replaced. The sample was then macerated with a sterile pestle, and 3 µl of proteinase K was added. The tube was incubated a 35 °C for one hour.

Subsequently, 200 µl of CTAB extraction buffer (2% CTAB, 2% PVP, 0.5% β-mercaptoethanol, 1,4 M NaCl, 20 mM EDTA, 10 mM Tris-HCL, pH 8,0) was added, and the sample was incubated a 65°C for 5 minutes. After homogenization for 1 minute, an additional 600 µl of CTAB buffet was added, followed by incubation at 65 °C for 1 hour. Then, 700 µl of chloroform: isoamyl alcohol (24:1) was added, and the sample was centrifuged at 14,000 rpm for 10 minutes at room temperature. The aqueous phase was transferred to a new tube. DNA was precipitated by adding 300 µl of cold isopropanol and 50 µl of 7,5 M ammonium acetate, and the sample was stored overnight at -20°C. DNA was recovered by centrifugation at 14,000 rpm for 20 minutes at 4°C. The supernatant was discarded, and the pellet was washed with 50 µl of 70% ethanol, followed by centrifugation at 14,000 rpm for 10 minutes at 4°C. The ethanol was discarded, and the pellet was air-dried and resuspended in 50 µl of TE buffer.

Partial sequences of the nuclear 28S ribosomal DNA (28S rDNA) and the mitochondrial cytochrome b (Cytb) gene of *Neobenedenia* spp. were amplified using the Polymerase Chain Reaction (PCR). Primers originally designed for *N. girellae* by Brazenor *et al.* (2018) were used successfully across the analyzed specimens. PCR reactions were carried out in a final volume of 25 µl. For 28S rDNA, the primers used were C1 forward (5'-ACCCGCTGAATTTAAGCAT-3') and EC-D2 reverse (5'- TGGTCCGTGTTTCAAGAC-3'); for Cytb, the primers were M1676 forward (5'-TGAGTTATTATTGATGTAGAGG-3') and M1677 reverse (5'- AAAATATCAKTCAGGCTTWA-3').

Cycling conditions varied depending on the genetic marker. For 28S rDNA, the thermal profile included an initial denaturation at 95 °C for 2 minutes, followed by 35 cycles of denaturation at 95 °C for 1minutes, annealing at 55 °C for 40 seconds, and extension at 72 °C for 1,2 minutes, with a final extension at 72 °C for 10 minutes. For Cytb, PCR began with an initial denaturation at 95 °C for 5minutes, followed by 5 cycles of 95 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 30 seconds, then 30 additional cycles of 95°C for 30 seconds, 55 °C for 1 minutes, and 72 °C for 1 minutes, ending with a final extension at 72°C for 10 minutes. PCR products were run on 1% agarose gels (SYNERGEL™), stained with Uview™ 6x loading dye, and visualized under a transilluminate (Chemi Doc™ MP Imaging System BIORAD). Amplified products were purified and sent for sequencing to by GENEWIZ and MCLAB (both in the USA).

Phylogenetic Analyses

Sequences were visualized using Chromas v2.6.6 (Technelysium Pty Ltd), edited, and exported in FASTA format for alignment and consensus generation. Consensus sequences of the 28S rDNA and Cytb obtained in this study were deposited in GenBank (Table 1). For phylogenetic comparisons, additional Cytb and 28S rRNA sequences of *N. girellae* and *N. melleni* were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/), with accession numbers ranging from MH843690 to MG193667 and HQ684800 to HQ684815, respectively. These sequences were aligned with our dataset using Clustal W implemented in MEGA X v10.2.6 (Stecher-Velasco *et al.*, 2020).

Phylogenetic analyses were performed using Maximum Parsimony (MP) and Maximum Likelihood (ML) methods in PAUP* 4.10b (Swofford, 2003), with node support evaluated through 1,000 bootstrap replicates. Bayesian Inference (BI) was conducted in MrBayes V3.1.2 (Ronquist & Huelsenbeck, 2003), using 3,000,000 generations and sampling every 100 generations. The best-fit nucleotide substitution model, TrN+I+G, was selected using ModelTest 3.07 (Posada & Crandall, 2001) under the Akaike Information Criterion (Akaike, 1974). Phylogenetic trees were visualized and edited in FigTree v1.4.4.

Additionally, pairwise p-distances within clades were calculated MEGA X and expressed as percentage differences by multiplying the p-distance values by 100. Node support in phylogenetic trees was evaluated using bootstrap values and posterior probabilities.

Ethical aspects: For the present study, the general procedure for animal welfare was followed based on the Mexican law on animal welfare (NOM-062-ZOO-1999, Technical Specifications for the Production, Care, and Use of Laboratory Animals). This regulation does not include aquatic organisms; however, the ethical standards described in the ARRIVE Guidelines (Animal Research: Reporting of In Vivo Experiments) were observed (Kilkenny et al., 2010).

RESULTS

Neobenedenia cibnorensis n. sp. Valles-Vega, Pérez-Urbiola & Ascencio

Phylum Platyhelminthes Minot, 1876
Class Monogenea Van Beneden, 1858
Order Capsalidea Lebedev, 1988
Family Capsalidae Baird, 1853
Subfamily Benedeniinae Johnston,1931
Genus *Neobenedenia* Yamaguti, 1963

Taxonomic summary

Type host: Almaco Jack (*Seriola rivoliana*)

Type locality: La Ensenada de La Paz, Baja California Sur, Mexico

Site of host: external body, in the skin

Prevalence: 100% (nine fishes examined)

Mean intensity: three adult parasites per host

Material deposited: Holotype, CPPNP 1395; Paratypes, CPPNP 1396, 1397 and 1398. Colección de Parásitos de Peces del Noroeste del Pacífico from Centro de Investigación en Alimentación y Desarrollo, Unidad Mazatlán, Mexico

GenBank accession numbers: See Table 1 (28S and Cytb) Etymology: The new species is named in honor of the Centro de Investigaciones Biológicas del Noroeste, recognizing it as an institution of higher education and research in Mexico

Morphological description

Description based on 28 specimens collected from La Ensenada de La Paz. Body elongated oval, total body length including haptor 4291,79 ± 976,52 (2581,0 – 5843,0), body width 2306,46 ± 613,26 (1369 – 3075) (Figs 2 and 3A). Anterior attachment organs disc-like, 353,9 ± 80,7 (158,8 – 476,4) long 351,99 ± 79,68 (178,65– 416,85) wide, unlobed, aseptate (Fig. 3B). Two pairs of eyes anterior to pharynx. Pharynx 335,0 ± 90 (179,0 – 496,0) long by 381,0 ± 88,0 (238,0 – 536,0) wide, with five lobes (Fig. 3 B). Haptor almost circular 1117,67 ± 243,77 (664,0 – 1495,0) long 1103,08 ± 277,83 (590 –1434) wide (Fig. 3C). Marginal membrane of haptor 73,0 ± 17,0 (44,0 – 93,0) wide. Accessory sclerites 189,81 ± 50,60 (125,0 – 250,0) long, strong, distally branched (Fig. 3 C, D). Anterior hamuli 316,67 ± 56,33 (225 – 400) long, relatively stout with sharp, recurved points (Fig. 3 C, D). Posterior hamuli 148,0 ± 11,0 (125,0 – 174,0) long, straight, poorly sclerotized with fine recurved points (Fig. 3C, D). Marginal hooklets 10,86 ± 0 (10,30 – 11,33) long arranged radially in haptor (Fig. 3D). The intestinal crura extends rearward to the body's end, concluding without an outlet. Testes spherical, 363,39 ± 108,54 (175,0 – 550,0) long 336,0 ± 98,0 (175,0 – 475,0) wide at midbody, postovarian, lobed, fenestrated, penetrated by several dorsoventral muscle bundles (Fig 3. E), in some specimens (n=10), it is observed that the length is similar to that of the ovary. Vas deferens travels sinistrally to the ovary and vitelline reservoir before entering the penis sac dorsally at the ootype level. Claviform cirrus complex, located behind the pharynx, consists of a muscular cirrus, prostatic reservoir, and seminal vesicle, all enclosed within the cirrus pouch (Fig 3. B, F). Ovary 271,0 ± 58,0 (179,0 – 357,0) long by 341,0 ± 93,0 (199,0 – 496,0) wide, medial and while the vagina is absent. Vitelline reservoir transversely wide, before the ovary (Fig 3. E). Ootype is positioned behind the cirrus pouch, with a short uterus connecting to the penis sac via the germiduct towards a shared genital opening located directly posterior to the anterior attachment organ. Vitelline follicles extend throughout the body. Vitelline follicles extend throughout the body. Eggs are tetrahedral, 130,0 ± 13,0 (105,0 – 146,0) long 111,0 ± 13,0 (82,0 – 128,0) wide, with two recurved appendages and a slender filament (Fig 3. F).

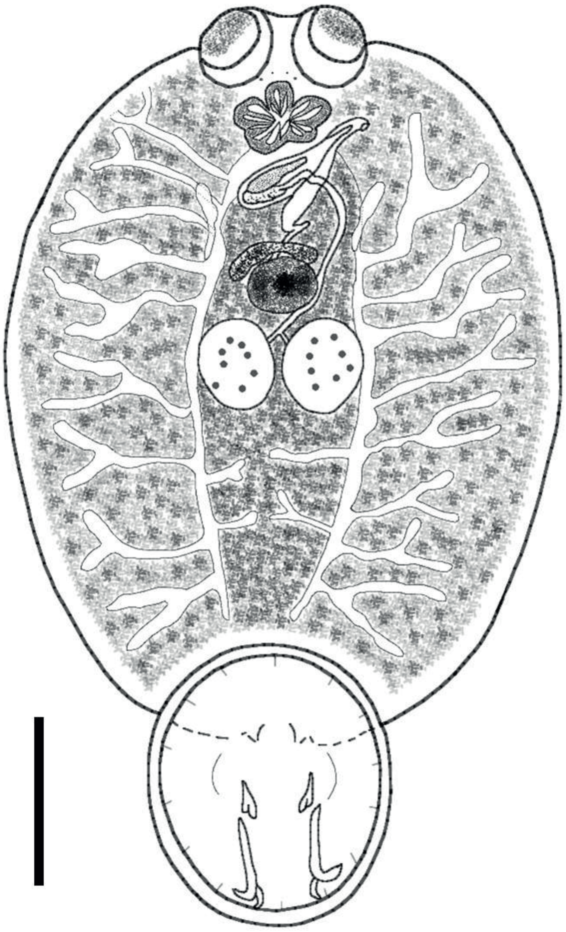

Figure 2. *Neobenedenia cibnorensis* n. sp., line drawings from *Seriola rivoliana*. Scale bar= 500µm.

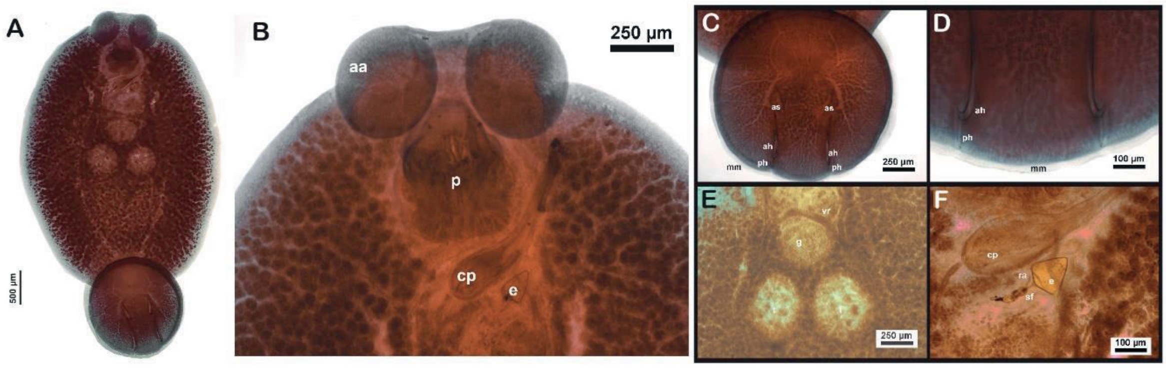

Figure 3. *Neobenedenia cibnorensis* n. sp., adult from *S. rivoliana* from La Ensenada de La Paz. (A) full-body view (B) anterior attachment organs with disc-like (aa), lobed pharynx (p) cirrus complex within cirrus pouch (cp) and egg (e). (C) the haptor with accessory sclerites (as), anterior hamuli (ah), posterior hamuli (ph) and marginal membrane (mm). (D) detailed view of the marginal membrane and the distal part of anterior and posterior hamuli. (E) central body region showing the testes (t), germarium (g) and vitelline reservoir (vr). (F) close-up of the egg showing two recurved appendages (ra) and a slender filament (sf), scale bar: A) 500µm; B, C & E) 250 µm; D) and F) 100 µm

Table 1. *Neobenedenia girellae* and *Neobenedenia cibnorensis* n. sp. by host species and location from Baja California Sur, Mexico, including GenBank accession numbers.

Host species	*Neobenedenia* species	Location	Genbank accession codes	
			28S	Cytb
Seriola rivoliana	*N. cibnorensis* n. sp.	Ensenada de La Paz	OQ418093	OQ988164
Seriola rivoliana	*N. cibnorensis* n. sp.	Ensenada de La Paz	OQ418094	OR022087
Seriola rivoliana	*N. cibnorensis* n. sp.	Ensenada de La Paz	OQ418095	OR022088
Seriola rivoliana	*Neobenedenia* sp.	Ensenada de La Paz	OQ418096	OR022089
Seriola rivoliana	*N. girellae*	Ensenada de La Paz	OQ418097	OR225614
Seriola rivoliana	*N. girellae*	Ensenada de La Paz	OQ418100	OR192970
Seriola rivoliana	*N. girellae*	Bahía de La Paz	OQ418098	OR192969
Seriola rivoliana	*N. girellae*	Los Cabos, BCS	OQ418099	OR184925
Seriola lalandi	*N. cibnorensis* n. sp.	Ensenada de La Paz	OQ418101	OR192968
Seriola lalandi	*N. girellae*	Bahía Magdalena	OQ418103	OR184924

Remarks

Capsalid specimens infecting *S. rivoliana* were assigned to the genus *Neobenedenia* based on key diagnostic features: two suckers on the haptor, dendritic cecal branches, very close or shared male and uterine pores, and the absence of a vagina (Whittington & Horton, 1996).

The genus *Neobenedenia* comprises eight species of monogeneans, two of which are considered cryptic due to morphological similarity (Whittington, 2004). *Neobenedenia cibnorensis* n. sp., is part of the *N. girellae* complex, which is considered cosmopolitan (Brazenor *et al.*, 2018). Interestingly, this species appears to infest various hosts species and has a wide geographic range, which is unusual since monogeneans typically exhibit host specificity and limited distribution due to biogeographic barriers. This condition may lead to environmental effects, such as temperature driving phenotypic plasticity in key taxonomic characters of *N. girellae* under certain conditions.

A more in-depth analysis like PCA (see Fig. 4) indicated that the two leading principal components account for a cumulative variance of 87.39%. Patterns of distinction between *Neobenedenia* taxa can be deduced from the first and second PCs. The PC1 correlates significantly with mean body length (r=0.97; Table 2), body width, and the width-length ratio (r=0.98; Table 2), indicating that the mean parasite size (i.e. width and length) is the main morphometric feature that distinguishes groups within *N. girellae*. Body proportions may also be used as discriminant features. For example, *N. girellae* on *Lates calcarifer* Bloch, 1790, is separated by its small size from monogeneans infesting other hosts (Fig. 4). Meanwhile, the newly described species (*N. cibnorensis* n. sp.) is the largest (Fig. 4), and has the highest width to length ratio, indicating a more rounded body shape (wl_ratio on Fig. 4 and Table 2).

The second principal component is closely associated with the sclerite length to body length ratio (r=0.97; Table 2).

Table 2. Numerical results of the Principal Component Analysis indicated as the percentage of cumulative variances (Cum. Variance), and the correlation coefficient of each Principal Component (PC) of the morphometric measurements: body length and width. The correlation of each PC with the width-length (wl_ratio), sclerites length to body length (sl_ratio), and the haptor to body length (hl_ratio) ratio are also indicated.

Principal Component	Cum. Vairance (%)	Length	Width	wl_ratio	sl_ratio	hl_ratio
PC1	66,33	0,97	0,98	0,81	-0,11	-0,87
PC2	87,39	0,03	0,12	0,21	0,97	0,24
PC3	98,24	-0,21	0,10	0,53	-0,22	0,40
PC4	99,86	0,13	0,13	-0,12	-0,04	0,18
PC5	100,00	0,05	-0,05	0,02	0	0,02

In this component, *N. cibnorensis* n. sp. is distinguishable from *N. girellae* by having larger sclerites relative to the body length, even when both species infest *S. rivoliana* (Fig. 4). Despite this distinction, it should be noted that the high variability of this proportion clearly exemplified in the specimens infesting *L. calcarifer* (blue dots in Fig. 4). This high variability may arise from the different salinity and temperature treatments used in the analysis done by the authors of the source of the data (REF).

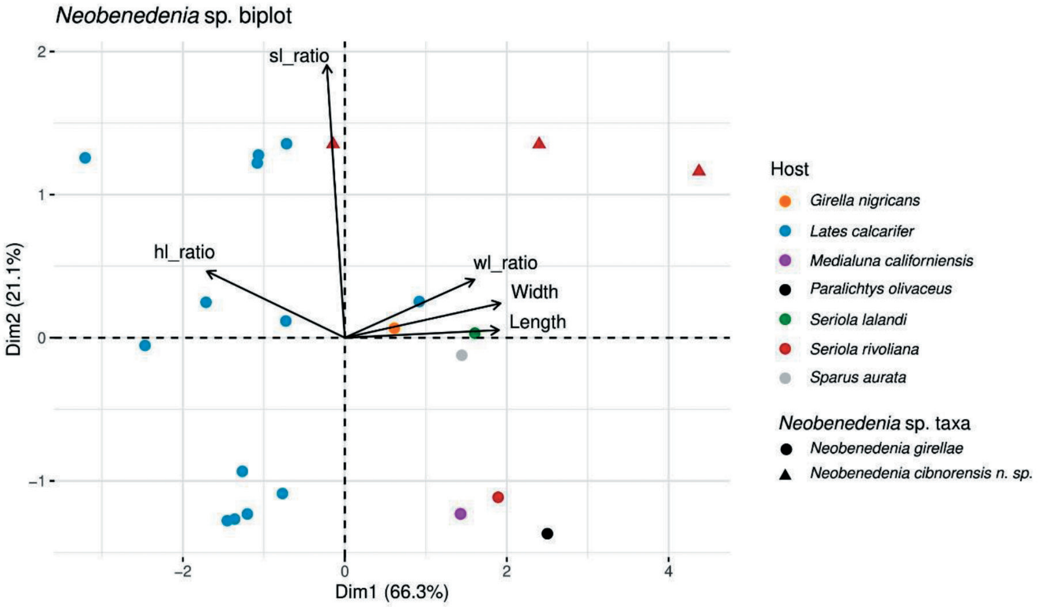

Figure 4. Biplot of the principal components analysis. Arrows' length indicates the contribution of morphometric variables to the principal components, while their angles show the degree of correlation (correlation coefficient). The percentage of variance explained by each component is indicated in the axis labels.

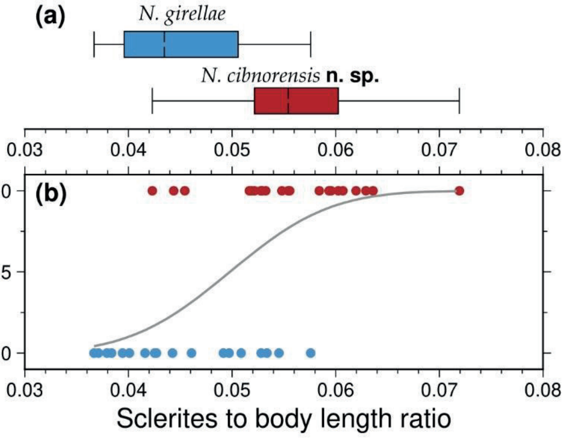

Figure 5. Comparison of the sclerites and body length relationships between *N. girellae* and *N. cibnorensis* n. sp. (a) Distribution of the sclerites/body length ratio (b) Logistic regression of the sclerites/body length

Given the exploratory nature of the PCA, the distinction between *N. girellae* and *N. cibnorensis* n. sp. was statistically corroborated by using an ANOVA and a logistic regression. Both analyses were based on the sclerites to body length ratio, which according to the second PC, is the morphometric feature distinguishing both taxa (Fig. 5a). Also, it was considered the phenotypic plasticity of some morphological characters observed in specimens of *Neobenedenia* when they are subject to different temperatures and salinities under experimental control (see for example *N. girellae* in *L. calcarifer*). For testing the statistical distinction between *N. girellae* and *N. ciboneresis* n. sp. was considered only specimens collected at La Paz for this study.

The mean of the sclerites length to body length ratio was 0.045 ± 0.07 (mean ± 1 sd) for *N. girellae* and 0.056 ± 0.007 for *N. cibnorensis* n. sp., a difference statistically significant at 95% of confidence (F [1,37] = 22.79, p-value=2.83x10^{-5}). This result confirms that *N. cibnorensis* n. sp. has on average, larger sclerites relation to body length. Thus, the sclerites to body length ratio is a useful morphological character for distinguishing *N. cibnorensis* n. sp. from *N. girellae*. In Figure 5b, the results of the logistic regression are presented, where it can be noted that a ratio above 0.054 have chance to be about 70 % to belong to *N. cibnorensis* n. sp. In contrast, a sclerites to body length ratio below 0.044 is unlikely bears to the new species, *N. cibnorensis* n. sp. (chance below 25 %; Figure 5b). Nonetheless, is evident that the range of this proportion overlaps between both taxa, highlighting that it is not a perfect discriminant feature.

Phylogenetic analysis

Phylogenetic analysis based on the 28S and cytochrome b sequences, using Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI), consistently supports *Neobenedenia cibnorensis* n. sp. as a distinct and monophyletic clade. In the 28S phylogeny (Fig. 6) recovered *N. cibnorensis* n. sp. within the same clade as *N. girellae*, with moderate support values (MP: 59%, ML: 75%, BI: 1.0), suggesting close phylogenetic affinity between the two taxa. However, the cytochrome b phylogeny (Fig. 7) revealed a different pattern, where *N. cibnorensis* n. sp. formed a distinct monophyletic clade with strong support (MP: 90%, ML: 89%, BI: 1.0), clearly separated from *N. girellae* and other *Neobenedenia* species.

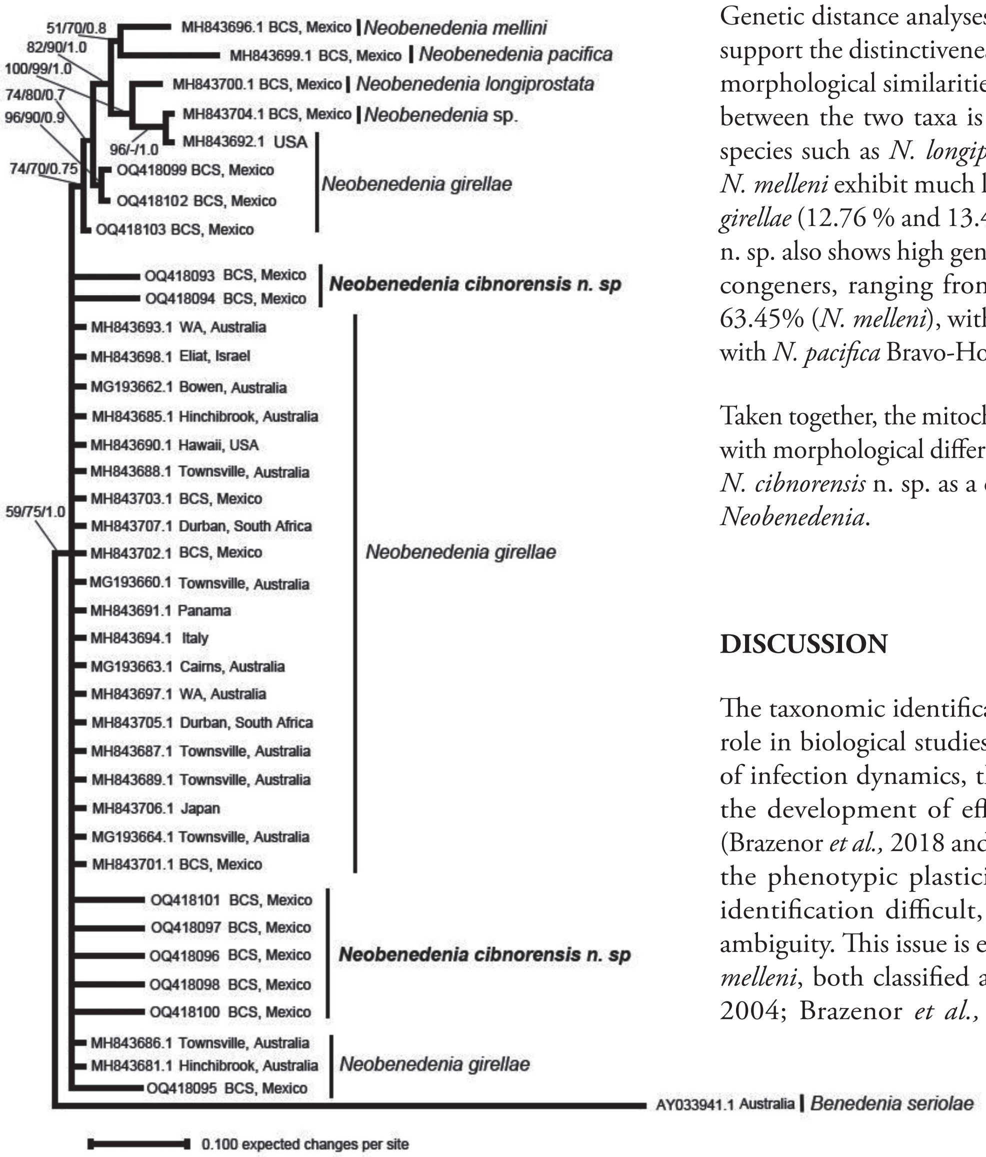

Figure 6. Phylogenetic tree based on sequences of the 28S gene of *Neobenedenia* spp., including *N. cibnorensis* n. sp. Values above the nodes represent Maximum likelihood/ Maximum parsimony/ Bayesian inference support.

This topological incongruence likely reflects the differing evolutionary rates of the two markers. While the conserved nature of the 28S rDNA may limit its ability to resolve recent divergences, the higher variability of cytochrome b provides better resolution at the species level.

Genetic distance analyses based on cytochrome b further support the distinctiveness of *N. cibnorensis* n. sp. Despite morphological similarities with *N. girellae*, the p-distance between the two taxa is 59,42%. In comparison, other species such as *N. longiprostata* Bravo-Hollis, 1971, and *N. melleni* exhibit much lower genetic divergence from *N. girellae* (12.76 % and 13.43%, respectively). *N. cibnoresnsis* n. sp. also shows high genetic distances with the remaining congeners, ranging from 58.47% (*N. longiprostata*) to 63.45% (*N. melleni*), with the highest divergence observed with *N. pacifica* Bravo-Hollis, 1971 (62.15%). See Table 3.

Taken together, the mitochondrial evidence, in combination with morphological differences, supports the recognition of *N. cibnorensis* n. sp. as a distinct species within the genus *Neobenedenia*.

DISCUSSION

The taxonomic identification of parasites plays a pivotal role in biological studies, facilitating the understanding of infection dynamics, the prediction of outbreaks, and the development of effective management strategies (Brazenor *et al.,* 2018 and reference therein). Nevertheless, the phenotypic plasticity of the species makes their identification difficult, often resulting in taxonomic ambiguity. This issue is exemplified by *N. girellae* and *N. melleni*, both classified as cryptic species (Whittington, 2004; Brazenor *et al.,* 2018). This study presents a new species, *Neobenedenia cibnorensis* n. sp., found in the Ensenada de La Paz in the Gulf of California. Our discovery is based on a comprehensive phylogenetic analysis that includes different isolates from various host fish species and countries registered previously by Brazenor *et al.* (2018) and Zhang *et al.* (2014).

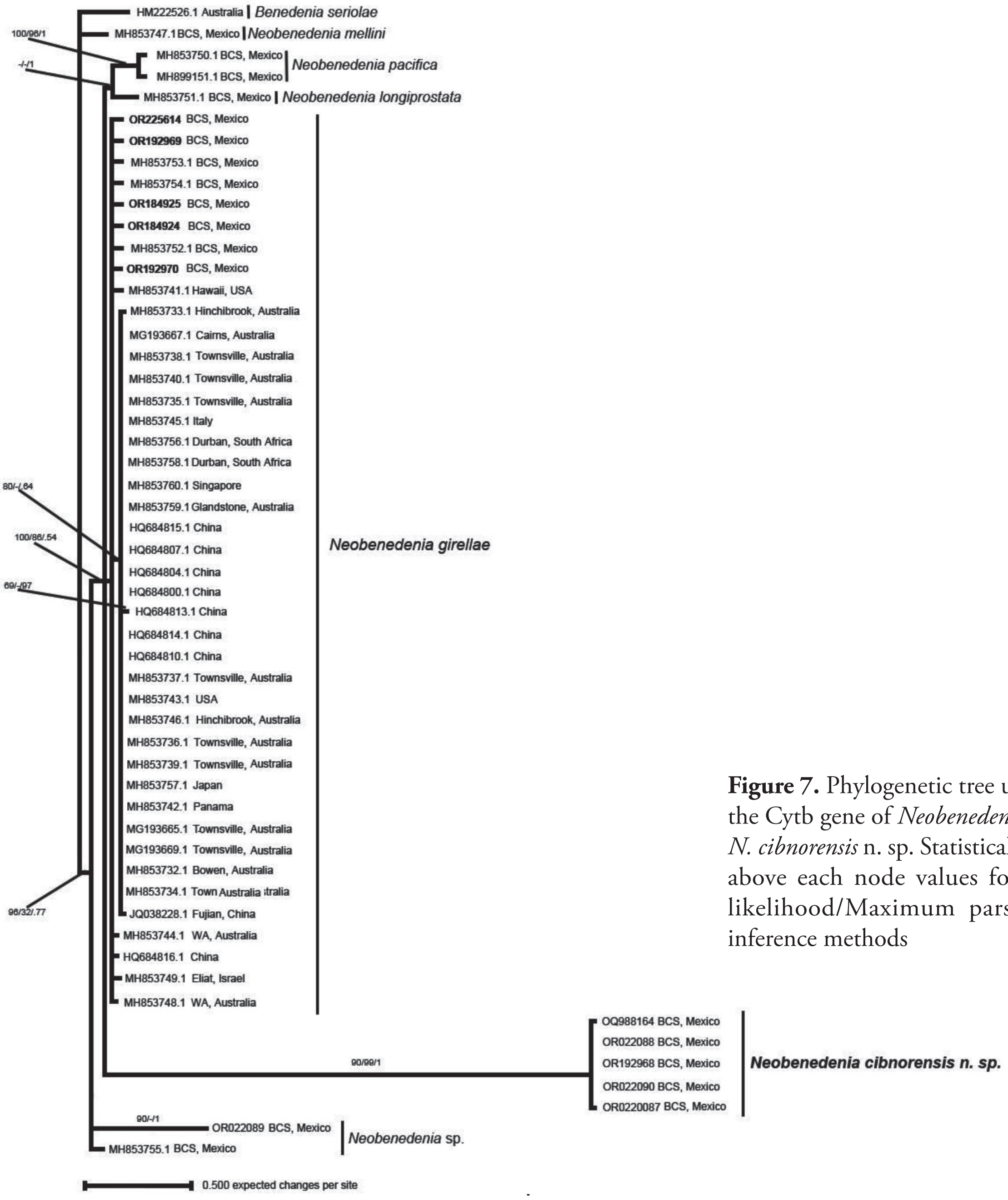

Figure 7. Phylogenetic tree using sequences of the Cytb gene of *Neobenedenia* spp., including *N. cibnorensis* n. sp. Statistical support is shown above each node values for the Maximum likelihood/Maximum parsimony/Bayesian inference methods

Table 3. Genetic divergence (percentage) of the Cytochrome b gene between *Neobenedenia* species.

*Neobenedeni*a species	*N. cibnorensis* n. sp.	*N. girellae*	*Neobenedenia* sp.	*N. pacifica*	*N. longiprostata*	*N. melleni*
N. cibnorensis n. sp.						
N. girellae	59,41					
Neobenedenia sp.	61,90	23,76				
N. pacifica	62,15	15,93	27,67			
N. longiprostata	58,47	12,76	25,94	16,50		
N. melleni	63,45	13,42	24,63	20,45	16,62	

Previous research suggested cryptic species within *N. melleni* based on 28S rDNA (Whittington, 2004; Whittington *et al.,* 2004). Subsequent analysis, encompassing nuclear and mitochondrial genes, confirmed genetic distinctions from *N. girellae* with *N. melleni*. Remarkably, *N. girellae* appears to infect various host species and has a wide geographic range, which is unusual as monogeneans typically show host specificity and limited dispersal due to biogeographic barriers. In our study, we used genetic markers with different resolution levels. Analysis of 28S rDNA suggests that the specimens we studied, classified as *N. girellae* following Brazenor *et al.* (2018), are associated with different host species in distinct geographical regions (Fig. 5). These findings may be attributed to the highly conserved segments of this gene, facilitating the identification of homologous sequences (Hillis & Dixon, 1991).

However, when examining genetic distances using Cytb between *N. girellae* and *Neobenedenia cibnorensis* n. sp. from La Ensenada de La Paz (as per Brazenor *et al.,* 2018) (see Figs. 5-6 and Table 2), it becomes evident that these are distinct species. This finding aligns with the description by Brazenor *et al.* (2018) based on specimens collected from lutjanid hosts in La Paz, Mexico. Additionally, Sepúlveda & González (2019) have reported other *Neobenedenia* species in the Chilean coast, specifically from *S. lalandi*. These differences may be indicators that *Neobenedenia cibnorensis* n. sp. diverged from *N. girallae*, a conclusion consistent with the results obtained from 28S rRNA analysis.

Morphometric analysis further corroborates these distinctions. Principal Component Analysis (PCA) demonstrated that key features, such as the width to length ratio and the scletire to body length ratio, are critical for distinguishing the species (Fig. 4; Table 2). *N. cibnorensis* n. sp. was characterized by larger body size, a more rounded shape, proportionally larger sclerites compared to *N. girellae*. Despite this, the overlap in the sclerite to body length ratio (Fig. 5b) highlights the limitations of morphological features as standalone taxonomic markers, especially in light of the phenotypic plasticity observed in monogeneans under different environmental conditions (Brazenor & Hutson, 2015; Hutson *et al.,* 2022). Environmental variables, particularly temperature and salinity, significantly influence parasite morphology, as evidenced by specimens from *N. girellae* reared under control conditions.

Statistical analysis, including ANOVA and logistic regression, validated the morphometric differences between the two species. For example, the mean sclerite to body length ratio was significantly higher in *N. cibnorensis* n. sp. (0.056 ± 0.007) than in *N. girellae* (0.045 ± 0.007), confirming the taxonomic distinction at a 95% confidence level ($F[1, 37] = 22.79$, $p = 2.83 \times 10^{-5}$). However, given the overlap in proportions and the potential influence of environmental changes on these traits, genetic differentiation remains the most reliable approach for distinguishing cryptic species (Whittington *et al.,* 2004; Zhang *et al.,* 2014).

Preliminary studies by Valles-Vega *et al.* (2019) further support the divergence between *N. cibnorensis* n. sp. and *N. girellae*, highlighting distinct thermal preferences and tolerances in *N. cibnorensis* n. sp. These thermal traits likely influence the ecological niches each species occupies and may have contributed to their evolutionary separation over time. Environmental factors, such as temperature, can significantly affect growth, reproduction, and overall fitness, potentially maintaining the isolation of these two species despite their overlapping habitats and host species. This evidence enhances our understanding of the biological and ecological distinctions between *N. cibnorensis* n. sp. and *N. girellae*, suggesting that thermal adaptability may be a key factor in their evolutionary trajectories.

Although the integrative approach combining morphometric and molecular analyses strongly supports the recognition of *N. cibnorensis* n. sp. as a distinct species, some limitations should be considered. The study was restricted to the southern Gulf of California, which may restrict the inference of the species' full geographic distribution and genetic diversity. Environmental factors, such as temperature and salinity, could also influence morphological traits and contribute to intraspecific variation. Additional studies considering temporal and environmental variation, as well as potential infestations in other host species, could help clarify phylogenetic relationships and the extent of phenotypic plasticity. Nonetheless, the combined evidence supports the designation of *N. cibnorensis* n. sp. as a distinct species.

Moreover, these findings indicate that La Ensenada de La Paz harbors previously undescribed species and that *S. rivoliana* is infested by two distinct parasites: *N. girellae* and *N. cibnorensis* n. sp., though their seasonal variability remains unknown. Another species described in this area is *N. pacifica*, further emphasizing the region's importance for *Neobenedenia* diversity. This makes La Paz, Baja California Sur, Mexico, a key location for studying host-parasite relationships and co-evolutionary dynamics. Additionally, evidence suggests that spatial patterns and mesoscale processes in the region influence populations within and beyond the Gulf of California (Saavedra-Soleto *et al.,* 2013), underscoring the significance of such studies. Future research should focus on assessing parasite diversity in the region to better understand how these interactions vary across hosts and environmental conditions.

This research represents the first documentation of a previously undescribed *Neobenedenia* species in Pacific Mexican aquaculture. Our findings highlight the crucial role of the Cytb gene in distinguishing *N. cibnorensis* n. sp., found in *S. rivoliana* at La Ensenada de La Paz, Mexico, from *N. girellae,* previously identified in the Pacific Ocean. We confirm that *S. rivoliana* hosts two distinct species: *N. girellae* in Bahia de La Paz and *N. cibnorensis* n. sp. in La Ensenada de La Paz.

The observed isolation of the parasite populations could potentially be attributed to oceanographic barriers such as density fronts, circulation patterns, or local processes such as tidal currents within the coastal lagoon. These findings underscore the importance of thorough taxonomic assessment and genetic analysis in understanding the diversity of parasites affecting aquaculture species, which can have significant implication for management and conservation strategies in marine ecosystems.

This study emphasizes the significance of employing combined morphometric and molecular approaches for species delimitation within *Neobenedenia*. The results support the development of biosecurity protocols for *Seriola* cultivation in the Gulf of California and may inform *Seriola* aquaculture in other regions, aiming to mitigate the impacts of parasites infestations.

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