Taxonomic revision, morphology, and genetic variability of *Holothuriophilus trapeziformis* Nauck, 1880 (Decapoda: Pinnotheridae) from the Pacific coast of Mexico.

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Abstract

Background. *Holothuriophilus trapeziformis* Nauck, 1880 is a holothurian-dweller Pinnotherid crab and represents one of the two species of the genus, which is distributed along the Pacific coast of America. Currently, only one morphological character separates both species because, since 1880, only the females were known. Furthermore, the original description of *H. trapeziformis* and its subsequent descriptions are incomplete or ambiguous and genetic information for this species does not exist. Our goal here is to describe for the first time the *H. trapeziformis* male morphology, discuss the morphological variations observed and clarify the taxonomic status of the species, and provide a genetic comparison based on the DNA barcoding.

Methods. We used the integrative taxonomy to re-describe *Holothuriophilus trapeziformis*, including a complete morphological description of the male and female. We also compared, the intraspecific morphological variability and conducted a genetic analysis based on comparing of the COI gene among different sequences of the related Pinnotheridae prepared by us and available public databases.

Results. *Holothuriophilus trapeziformis*, as any decapod, has a strong sexual dimorphism. Fifty-five specimens collected on the Pacific coast of Mexico were examined, and the DNA barcodes were compared. *H. trapeziformis* is confirmed as a different species from *H. pacificus* by the general shape of the carapace, the previously known interdactilar gape condition, the ornamentation of the pincers fingers, and by the shape of the male abdomen and its first gonopod, also the interspecific COI divergences are >3%. Morphological variations coincide with COI, and a haplotype network resolution defined one clade and two subgroups. Genetic analyses determined a structure population with 22 haplotypes among regions with a gene flow of the possible ancestral haplotype from south to north. An emerging allopatric differentiation process is showed by both the species morphology and barcoding. Results coincided with the Barcode Index Number (BIN)

assigned to this species (BOLD: ADE9974). Moreover, *H. trapeziformis* is recorded for the first time within the intestine of its host, the sea cucumber *Holothuria* (*Halodeima*) *inornata* Semper, and its distribution range on the Mexican Pacific coast was extended.

Introduction.

Pinnotherids (Crustacea: Pinnotheridae) are true decapod crabs, which show a conspicuous sexual dimorphism, notably different morphological states of development and complex ecological relationships with different invertebrates, and can also be found in free life (Schmitt et al. 1973; Ocampo et al. 2011; Becker & Türkay 2017). Thirteen species are known to be endobiotic with sea cucumbers (Ng & Manning 2003). Of them, *Holothuriophilus trapeziformis* Nauck, 1880, is one of the two species of pinnotherids crabs described for the genus (Manning 1993); however, ~~his~~ its taxonomic status remains incomplete because male morphology is unknown and the available information about the female illustrations shows some inconsistencies. These situations have caused the differentiation of *H. trapeziformis* from *H. pacificus* ~~both species to be~~is based on a single morphological character ([please indicates the character here]~~(~~Campos, Peláez-Zárate & Solís-Marín 2012) that could be subjective. It should be added that, to date, there is no genetic information related to *H. trapeziformis*.

Holothuriophilus Nauck, 1880 from the Pacific coast of America~~, was established with~~ consists of *H. trapeziformis* from Mazatlan, Mexico, and ~~includes~~ *H. pacificus* (Poeppig, 1836*)* from Talcahuano, Chile (Manning 1993). Both species are associated with sea cucumbers (Garth 1957; Campos, Peláez-Zárate & Solís-Marín 2012). Also, *Pinnaxodes mutuensis* Sakai, 1939, from Aomori Bay, Japan (Takeda & Masahito 2000) and *P. tomentosus* Ortmann, 1894, from Brazil have been considered as belonging to *Holothuriophilus* (Melo & Bohes 2004; Ng, Guinot & Davie 2008). However, their definitive status is currently under revision due to differences in

コメントの追加 [TN1]: Do you mean two species are described under as new species of *Holothuriophilus*? *H. trapeziformis* is the only species described as a new species of the genus. *H. pacificus* was originally described as *Leucosia*.

コメントの追加 [TN2]: When compare with what?

書式変更: フォント : 斜体, コンプレックス スクリプト用のフォント : 斜体

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diagnostic characters and for being associated with mollusks as a host (Campos, Peláez-Zárate & Solís-Marín 2012).

Holothuriophilus is diagnosed by its transversally elongated carapace, wider anterior to middle portion; its short, robust and compressed walking legs, with the dorsal margin cristate; and the third maxilliped, with the ischiomerus indistinguishably fused (Garth 1957; Manning1993; Ng & Manning 2003; Campos, Peláez-Zárate & Solís-Marín 2012).

So far, *Holothuriophilus trapeziformis* can only be differentiated from *H. pacificus* based on a single morphological character and by an ecological condition related to the host specificity. The former species has a narrowed opening when the pincers' fingers are closed and its ecological host is the sea cucumber *Holothuria* (*Halodeima*) *inornata* Semper, but in the latter species the finger's gap is conspicuous and its host corresponds to *Athyonidium chilensis* (Semper) (= *Eucyclus chilensis*), another sea cucumber (Garth 1957; Campos, Peláez-Zárate & Solís-Marín 2012; Honey-Escandón & Solís-Marín 2018).

Also, Nauck (1880) did not designate a holotype when he described *H. trapeziformis*, the original description did not provide enough information, and the host identity was erroneously determined. Moreover, the female syntypes were deteriorated over time and the male was unknown (Bürger 1895; De Man 1887; Ng & Manning 2003). Later, Manning (1993), Ng & Manning (2003) and Ahyong & Ng (2007) examined the syntype series to complete the diagnosis and designated a lectotype which was described and illustrated. However, there are inconsistencies between their illustrations and the diagnostic characters are not informative when considering the information available for *Holothuriophilus pacificus*. In addition, for 84 years *H. trapeziformis* had not been collected again until Caso (1958, 1964, 1965). She collected four pinnotherids determined as *Pinnixa barnharti* (not *Pinnixa barnharti* Rathbun, 1918) associated with *Holothuria inornata Semper* and *H. kefersteinii* (Selenka) (= *H. riojai* Caso, 1964). Thirty-

コメントの追加 [TN3]: Campos et al. (2012) did mentioned that "The taxonomy and systematics of these species is currently being studied by the first author and will be published elsewhere.", but they did not made any actions on these two species, so they should also be treated as *Holothuriophilus* in this paper.

コメントの追加 [TN4]: Sentences from here forward are detailed version of previous sentences. Need to revise the structure to make it in a consice manner.
Also need to indicate exactly what is the aim of this study.

four years later, one of Caso's specimens was determined as *Holothuriophilus* sp. by Campos, Díaz & Gamboa-Contreras (1998). More recently Campos, Peláez-Zárate & Solís-Marín (2012) updated the species diagnosis and made a review of the genus. Finally, Honey-Escandón & Solís-Marín (2018) confirmed the ecological association between *H. trapeziformis* and *Holothuria inornata*, but Caso's (1958, 1965) records of *Holothuria kefersteinii* as a host remains uncertain because the field collection data does not correspond with the material reviewed by Honey-Escandón & Solís-Marín (2018), and the location of these pinnotherids and their holothurian hosts is unknown (F Solís-Marín, 2018, pers. comm.).

For *Holothuriophilus trapeziformis* there is currently no data on genetic information and on its historical demography, contrary to *H. pacificus* that has information related to the COI gene sequence for one specimen from the shoreline in southern Chile (CFAD062-11; boldsystems.org). In this context, sequencing of approximately 650 bp region of the mitochondrial Cytocrome Oxidase 1 gene (COI) has been promoted to conform a standardized DNA barcode system with the aim of being one more tool for the identification of biological species with many applications in diverse fields of knowledge (Hebert et al. 2003; Hajibabaei et al. 2007). In spite of the difficulty to work with COI regarding the debate about the acceptance of one molecular marker as an accurate character to delimit a species (Will & Rubinoff 2004), it has been considered the best marker for identification in other decapods (Spielmann et al. 2019) and the utility of the DNA Barcoding (COI sequence) has been useful to delimit other pinnotherids (Ocampo et al. 2013; Perez-Miguel et al. 2019), brachyuran larvae (Brandão et al. 2016), and other crustacean taxa (Costa et al. 2007; Matzen da Silva et al. 2011).

Considering that integrative taxonomy based on morphological and molecular data, is increasingly useful to define and delimit biological species with greater certainty, the goal of this study is, therefore, to define the taxonomic status of *Holothuriophilus trapeziformis* by

completing the information on the species with the description of the male, revising of the morphological variability in both sexes, updating the range of distribution, and establishing a baseline of genetic variability and historical demography based on the mitochondrial COI gene. Finally, this information will provide new diagnostic characters that will allow a clearer separation of both species of *Holothuriophilus* from the Pacific coast of America.

Material & methods

Morphology

Fifty-two crabs belonging to the *H. trapeziformis* were extracted from the coelom and intestine of the sea cucumber *Holothuria inornata.* Hosts were manually collected through skin and SCUBA diving at a maximum depth of 10 meters in Sinaloa, Guerrero, and Oaxaca, Mexico. The collected material was labeled and fixed according to the Elías-Gutiérrez et al. (2018) protocol for the tissue preservation. Furthermore, due to the size of the specimens and the thickness of the cuticle, the preservative was injected into the body of the crabs and the hosts with individual insulin syringes to preserve DNA quality for subsequent molecular studies described later in the molecular data section.

All biological material (Table S1) was classified and deposited in the Scientific Collection of Marine Invertebrates of the Laboratorio de Sistemática de Invertebrados Marinos (LABSIM) from Universidad del Mar (UMAR), Oaxaca, Mexico (OAX-CC-249-11). Hosts were identified with specialized literature (Solís-Marín et al. 2009; Honey-Escandón & Solís-Marín 2018).

For the analysis of the taxonomic status of *Holothuriophilus trapeziformis* specialized literature from Nauck (1880), Manning (1993), Ng & Manning (2003), Ahyong & Ng (2007), and Campos, Peláez-Zárate & Solís-Marine (2012) was reviewed. Likewise, for *H. pacificus,* Poeppig (1836), Nobili (1901), Rathbun (1918) and Garth (1957), were reviewed.

Field permit for collections with non-comercial scientific research purposes was issued by Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA) and Comisión Nacional de Acuacultura y Pesca (CONAPESCA) (Collecting permit: PPF/DGOPA-301/17).

The species description follows the terminology of Campos et al. (2012) and Davie, Guinot & Ng (2015), this last one mostly for the general shape of the carapace, and setae terminology of Garm & Watling (2013). Drawings were made with the help of a lucid camera and then digitalized in a vector format. Pictures were taken with a digital camera Nikon D5100. Measures are given in millimeters and the latitude and longitude were obtained from Google Earth™.

Because we were only able to obtain nine specimens (three males and six females) from the type locality, in contrast to 47 (six males and 41 females) from the southern region, and due to morphological variability observed among individuals of the same sex and between them, as well as within and among geographic regions, it was necessary to standardize the observations of the variation with specimens at the same stage of development. The shared stage of development between the three regions (Sinaloa, Guerrero, and Oaxaca) corresponded to males and ovigerous females with a carapace width measurement equal to eight millimeters. To standardize the observations, the specimen and the dissected pieces were mounted on a plastic clay base to make the drawings. Punctually, for the carapace contour, these were mounted in such a way that the dorsal view of the posterior margin line of the carapace still can be observed. For the Mxp3, an attempt was made to extract it from its base to obtain both endopod and exopod, and to mount it with the articles in the same perspective. The cutting edge of the fingers chelae were cleaned of dirt in order to view all the teeth. The first gonopod was extracted from its base and the setae cleaned of dirt.

Abbreviations used in the text: CL, carapace length (taken as the middle line from the frontal margin to the posterior margin of the carapace); CW, carapace width (measured in its medium-anterior portion); Mxp2, second maxilliped; Mxp3, third maxilliped; P2–5, walking legs 1 to 4. Acronyms used in the text: BOLD, barcode of life database (boldsystems.org); BIN, barcode index number (*sensu* Ratnasingham & Hebert, 2013); BOLD-ID, Specimen ID in the Barcode of Life Data System; CNE-ICML-UNAM, National Collection of Echinoderms of the Institute of Marine Sciences and Limnology of the National Autonomous University of Mexico; DC-NHM, Division of Crustacea, Natural History Museum, Smithsonian Institution; SMF-ZMG, Senckenberg Museum für Naturkunde, Zoologisches Museum Göttingen University, Humboldt Universität, Berlin; UABC, Autonomous University of Baja California, Mexico; UMAR, Universidad del Mar campus Puerto Angel, Oaxaca, Mexico.

Collectors: AEV, Aidé Egremy Valdés; AGF, Andrea Glockner Fagetti; CCA, Carlos Cruz Antonio; AHM, Adanely Hernández Muñoz; FBV, Francisco Benítez Villalobos; FCC, Fernando Cortés Carrasco; HMC, Humberto Mesa Castillo; KFL, Karen Lizbeth Flores López; KMB, Karen Mesa Buendía; RGF, Rebeca Granja Fernández; VCH, Valeria Chavez García.

DNA extraction and PCR amplification

From the biological material collected in the field and some other taken from the OAX-CC-249-11 collection, genomic DNA was extracted from different tissue samples. For the crabs, muscle of the walking legs, chelae, or eggs were used. For the sea cucumber hosts, underlying muscle from the dorsolateral body wall and/or internal longitudinal ventral muscle were used. Tissues were placed into 96-well microplates with a drop of 96% ethanol, and DNA extraction was carried out following the standard glass fiber method of a mix of Proteinase K with invertebrate lysis buffer according to Ivanova, De Waard & Hebert (2006). Following DNA extraction, the PCR mixture with a final volume of 12.5 µl, contain 2 µl of Hyclone ultrapure water (Thermo

Fisher Scientific), 6.25 µl of 10% trehalose (previously prepared: 5 g D-(+)- trehalose dihydrate (Fluka Analytical) in 50 ml of total volume of molecular grade ddH2O), 1.25 µl of 10X PCR Platinum Taq buffer (Invitrogen), 0.625 µl of 50 µmol/L MgCl2 (Invitrogen), 0.0625 µl of 10 µmol/L dNTP (KAPA Biosystems), 0.125 µl of each 10 µmol/L primer, 0.06 µl of PlatinumTaq DNA polymerase (Invitrogen) and 2 µl of DNA template. All specimens were amplified with the Zooplankton primers (ZplankF1_t1 and ZplankR1_t1, see Prosser *et al*., 2013 for details). The reactions were cycled at 94°C for 1 min, followed by five cycles of 94°C for 40 seconds, 45°C for 40 seconds and 72°C for 1 min, followed by 35 cycles of 94°C for 40 seconds, 51°C for 40 seconds and 72°C for 1 min, with a final extension of 72°C for 5 min. PCR products were visualized on a pre-cast 2% agarose gels (E-Gel© 96 Invitrogen), and the most intense positive products were selected for sequencing.

Sequencing and DNA barcode

Selected PCR products were sequenced using a modified (Hajibabaei et al. 2005) BigDye© Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystem, Inc.), and then sequenced bidirectionally on an ABI 3730XL automated capillary sequencer using M13F and M13R sequence primers at the Biology Institute at the National Autonomous University of Mexico and at the Eurofins Genomics Louisville Laboratory. Sequences were edited using CodonCode© v 3.0.1 (CodonCode Corporation, Dedham, MA, USA) and uploaded to BOLD. In some cases, the original forward and reverse tracers uploaded to BOLD were checked again, consensus assembly was generated, and edited manually with Sequencher© 4.1.4. (Gene Codes Corporation, Ann Arbor, MI, USA), and then they were aligned using BioEdit© (Hall 1999).

Phylogeny and distance analysis

COI sequences generated for *Holothuriophilus trapeziformis* in this study were compared with COI sequences from other pinnotherids collected in the Eastern Pacific coast of America,

224 considered as an outgroup and available in BOLD and/or GeneBank (Table S2). Sequence data,
225 trace files, and primer details for all *H. trapeziformis* specimens and for the outgroup species are
226 available under the dataset name PINMX1HT ("Htrapeziformis from Mexico"; DOI:
227 dx.doi.org/10.5883/DS-PINMX1HT) in the Barcode of Life Data System (barcodinglife.org).
228 Additionally, *Holothuriophilus trapeziformis* sequences were uploaded to GenBank
229 (https://www.ncbi.nlm.nih.gov/).
230 To infer the phylogenetic relationships, the best-fitting evolution model of nucleotide substitution
231 for distance based on COI alignments was established on the Maximum Likelihood (ML) for 24
232 different nucleotide substitution models, selected according to the Akaike (AIC) and Bayesian
233 (BIC) criterion (Darriba et al. 2011), and tested using jModelTest© 2.1.10 (Posada & Buckley
234 2004). The final phylogenetic topology was obtained with nodal support for the resulting
235 branches estimated with 1000 bootstrap replicates in MEGA© 6.0 (Tamura et al. 2013). Finally,
236 the resulting topology was edited, and a simplified tree was constructed using FigTree© (Rambaut
237 2016). Also, interspecific COI genetic distances for the dataset were estimated using the Kimura-
238 2 parameters distance method in MEGA© 6.0 (Tamura et al. 2013). Values greater than 3% were
239 considered as threshold for the delimitation of species (Hebert et al. 2003).
240 **Intraspecific DNA polymorphisms and historical demography**
241 In order to determine the genetic variation within the *Holothuriophilus trapeziformis* group, 37
242 sequences from the Pacific coast of Mexico were aligned with MUSCLE routine, and
243 intraspecific genetic divergences were obtained using the Kimura 2-parameter substitution model
244 with a bootstrap method in MEGA© 6.0 (Tamura et al. 2013).
245 Genetic diversity was estimated with the haplotype diversity (Hd), number of haplotypes (H),
246 nucleotide diversity (π), number of polymorphic or segregating sites (S), average number of
247 nucleotide differences between pairs of sequences (k), guanine and cytosine content (G+C), and

all the haplotypes of the genetic variants of COI within species were obtained using DnaSP© v6.12.01 (Rozas et al. 2003, 2017) (http://www.ub.edu/dnasp/).

The genetic differentiation between localities (Sinaloa, Guerrero, Oaxaca) was analyzed with the aid of a fixation index Phist (Φ_{ST} test; with a 10000 permutations for pairwise genetic distance) using Arlequin© v3.5.2.2 (Excoffier & Lischer 2015) (http://cmpg.unibe.ch/software/arlequin35/). Phist values ranged from 0 to 1, and can be interpreted according to a scale range of 0.05 as low, 0.05 to 0.15 as moderate, 0.15 to 0.25 as great, and above 0.25 as very great; though there is no strict consensus about that scale range (Hartl & Clark 1997). This metric considers the haplotype phylogenetic distance and is more robust when the genetic diversity within the localities increases (Munguia-Vega et al. 2014). Additionally, the spatial relationships between the sampled localities were analyzed through the Spatial Analysis of the Molecular Variance (SAMOVA) evaluating the most likely number of groups ranging from k=1 to K=2, and significance of Φ statistics was tested by 100000 permutations using SAMOVA 2.0 software (Dupanloup et al. 2002). The correlation between genetic divergence and geographical distance among localities (isolation by distance) was tested with a Mantel test performed in XLSTAT v. 2020.1 (https://www.xlstat.com/en/), the p-value was estimated by 10000 Monte Carlo simulations.

Graphical explanation for biogeographical relationships of COI sequences was represented with a PopART haplotype network (http://popart.otago.ac.nz/index.shtml) considering the parsimony criterion (Clement et al. 2002; Leigh & Bryant 2015).

Mismatch distribution obtained with DnaSP© was used to deduce if a population has undergone sudden population expansion; a unimodal distribution indicates recent population expansion with little lineage loss, whereas no defined multimodal distribution indicates a constant size growth with stochastic lineage loss (Harpending et al. 1993). A goodness of fit between the mismatch

distributions was tested under a coalescent model with the Raggedness index (r) and R_2 function in Arlequin©, because these are considered powerful metrics to determine population change when sample size is small (Harpending 1994; Ramos-Onsins & Rozas 2002). In order to examine the signature of population demographic changes in *Holothuriophilus trapeziformis* sample, we used the Tajima's D statistic with a 1000 coalescent permutations to infer if the data conformed to expectations of neutrality model or if it departed from them; where a statistic near to zero indicates a constant-size population, significant negative values indicate a sudden expansion, and significant positive values indicate recent population bottleneck or a population subdivision (Ramos-Onsins & Rozas 2002).

Results

Here we analyzed the morphology of 56 specimens coming from three coastal regions in the Mexican Pacific in which the type locality is included; of them, only 51 were processed for the molecular analysis. All the material is listed in Table S1. Detailed morphological revision allowed us to determine notable variations, mostly on the carapace general shape, features of the first male gonopod, and in the pincers chelae ornamentation. Northern type locality morphology shows a notable variation in the general carapace outline shape and general appearance which looks more stout and eroded with respect to that of the southern specimens; however, all specimens show features that define *Holothuriophilus trapeziformis* according to Ng & Manning (2003), Campos, Peláez-Zárate & Solís-Marín (2012). Besides that, previously undescribed structures like the Mxp2 and male second gonopod plus the genetic data resolution, confirm that all the revised material corresponds with the *H. trapeziformis*. Complete morphology description

of the male and discussion of character variations in both sexes are annotated in the Systematic section and genetic analyses are annotated after that.

Systematics

Infraorder Brachyura Latreille, 1802

Family Pinnotheridae De Haan, 1833

Genus *Holothuriophilus* Nauck, 1880

Holothuriophilus. — Manning, 1993: 225 ~~(First genus diagnosis)~~.

Diagnosis (modified from Manning 1993). Carapace broader than long, **widest on mid anterior portion, transversely** subrectangular**, subovate or subtrapezoidal.** Third maxilliped with ischium and merus indistinguishable fused; exopod with **one segmented** flagellum; **endopod** palp 3-segmented; propodus shorter than carpus, conical; subspatulate dactylus articulates basally on propodus, extending beyond end of propodus. Dactyli of walking legs similar and subequal, short. Abdomen of seven segments in both sexes.

***Holothuriophilus trapeziformis* Nauck, 1880**

(Figs. 1A–G, 2A–D, 3A–F 4A–F, 5A–D)

Holothuriophilus trapeziformis Nauck, 1880: 24, 66 (Brief diagnosis of cephalothorax and Mxp3, type locality Mazatlan, Mexico, indicates association with *Holothuria maxima* Semper).—De Man 1887: 721–722 (Female syntype redescription, CW = 13.8 mm, CL = 10.5 mm).—Manning 1993: 524–528, Fig. 3C (Reinstates and diagnoses of the genus).—Ng & Manning 2003: 903, 916-918, Fig. 7C–F, (Designates female lectotype: SMF-ZMG 67/565a, CW = 7.7 mm, LC = 4.8 mm, illustration of Mxp3 and walking legs).—Ahyong & Ng 2007: 213-214, Fig. 20, (Illustrate the general shape of the body, cheliped and Mxp3 of the lectotype SMF-ZMG 170 (Go565a), CW = 7.7 mm, CL = 4.8 mm).—Campos, Peláez-Zárate & Solís-Marín 2012: 57–62, Figs. 1A, B, 2A–D (Record specimens from Punta Tiburon, Mazatlan, Sinaloa, Mexico, associated with

コメントの追加 [TN5]: What do characters highlighted by bold mean???

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Holothuria lubrica and *H. inornata* and from Ixtapa, Guerrero, Mexico, associated with *Holothuria kefersteinii* and *H. inornata* deposited in the CNE-ICMyL-UNAM Collection). *Pinnotheres trapeziformis* Bürger 1895: 380–381, plate 9, Fig. 26, plate 10, Fig. 25 (Brief description of one female (CW = 14 mm, CL = 10 mm) associated with *Holothuria maxima* Semper from unknown locality, and one specimen erroneously determined as a male (CW = 5 mm, CL = 8.5 mm) associated with *Holothuria inornata* Semper, from Mazatlan, Mexico).—Adensamer 1887: 107 (Provides the catalog number for the SMF).—Tesch 1918: 285 (list of species).—Schmitt, McCain & Davidson 1973: 5, 13, 89 (Annotated checklist of the Pinnotheridae from the DC-NHM).

Pinnoteres trapeziformis Balss 1957: 1417 (not 1956 *fide* Schmitt, McCain & Davidson 1973).

Pinnixa barnharti (no Rathbun, 1918) Caso 1958: 329 (First record since Nauck (1880) of a specimen from playa El Almacen, Guerrero, identified by Rioja), 1965: 254–26 (Second record of three specimens from playa Las Gatas, Guerrero, identified by Rioja).

Holothuriophilus sp. Campos, Díaz & Gambóa-Contrera 1998: 377, Fig. 1E (Corrects as *Holothuria* sp. the name of a *Pinnixa barnharti* specimen recorded by Caso (1965) from Guerrero, illustration of the Mxp3).

Material examined: 56 specimens: 25 ovigerous females, 22 females, nine males (Table S1).

General distribution: Tropical Eastern Pacific (Mexico).

Previous records: Mazatlan, Punta Tiburon (Sinaloa); Ixtapa (Guerrero).

New records: playa Pinitos (Sinaloa); playa Nudista, playa Zacatoso, playa Caleta de Chon (Guerrero); playa Agua Blanca, playa Coral, playa Camaron, playa Panteón, playa Estacahuite, playa La Tijera, bahia San Agustin, playa El Tejon (Oaxaca).

BIN: BOLD:ADE9974

コメントの追加 [TN6]: 1. Don't include memo of literature in synonymy list.
2. Including these literature in synonymy list means that you consider these records under respective names are positively of H. trapeziformis.

Size range (mm): Males: CW = 5.5–11, CL = 3.2-7; females: CW = 5.1–11, CL = 3–7; ovigerous females: CW = 7.3–13, CL = 5–8.

Diagnosis (modified from Campos, Peláez-Zárate & Solís-Marín 2012). Carapace transversally elongated, widest anterior to middle portion, broader than long, **general shape transversely suboval or subtrapezoidal**; anterolateral margin cristate, **with a hepatic notch which has a vanished blunt tooth, notch sometimes visible only in lateral view**; front under postfrontal ridge, deflexed, emarginated, its margin scarcely visible in dorsal view. Third maxilliped with ischium merus indistinguishably fused, palp 3-segmented; carpus subequal in length to subtrapezoidal propodus; spoon-shaped **or suboblong** dactylus articulated on medial ventral third of propodus; tip of dactylus slightly overreaching tip of propodus; exopod with one-segment flagellum. Cheliped merus, carpus **inner surface** densely setose; **propodus ventral inner margin with a row of short seta**; propodus and dactylus almost meeting when closed; **dactylus cutting edge with proximal denticles, a conspicuous tooth and a distal convex or acute projection**. Walking legs robust, similar in shape, segments compressed, **dorsal surface cristate; merus dorsal surface of W1, 3 and 4 with setae, W2 without seta;** carpi and propodi subequal; dactyli shorter than preceding articles, similar and subequal, last pair shorter than preceding. Abdomen with 6 somites plus free telson; **on male, margin of somite 4 to 6 concave, telson subrounded. Male first gonopod notably curved outward from its mid-distal portion.**

Description: Male (Fig 1A, B, C; UMAR-DECA-308; CW = 11 mm, CL = 7 mm): Carapace, transversely subtrapezoidal, wider than long, CW/CL ratio ca. 1.4 to 1.6, mid-anterior portion wider; anterolateral margins slightly projected, cristated, a hepatic notch with a blunt middle tooth (Figs. 2A, B; bold arrow); dorsal surface convex, smooth, without defined regions; mid-posterior and posterolateral surface with microscopic pits of variable size and pilosity (Figs. 2A, C; hollow circles and dots); inferior lateral margin with abundant plumose setae (Fig. 2A; simple

コメントの追加 [TN7]: Species diagnosis is a list of characters that separate its species from congeners. Are you sure all these characters are not shared with congeners?

コメントの追加 [TN8]: I guess redescribing this species is one of the major task of this study. You should redescribe it by yourself.

コメントの追加 [TN9]: Difficult to read – add line breaks accordingly.
What are female characters?

コメントの追加 [TN10]: How many males?

lines represent the enlarged schematic setae). Front bilobed, scarcely visible in dorsal view, margin granulated, surface slightly pubescent (Fig. 2B; dots). Orbits small, completely filled by eyes; eyes pigmented; ocular peduncle scarcely pubescent. Antennules robust; peduncle 2-segmented, biflagellate, transversely folded into the fossae; superior flagellum 2-articles, second article the longest, tapering distally, with six apical setae (Fig. 3Ba); inferior flagellum conic, with four articles decreasing in size, article one to three with a transverse line of simple setae, fourth article with two transverse lines of simple seta (Fig. 3Bb). Antennae long, slender, with 12 articles denuded of setae, last article with short apical setae (Fig. 3A). Pterygostomian region pubescence (Fig. 2B; fine dots). Buccal frame trapezoidal, completely covered by ~~the~~ Mxp3. Mxp2 endopod 5-articles, with setae (Fig. 3Ca), dactylus subrounded and shorter than propodus (Fig. 3C; black arrowhead); exopod 1-article, wider distally, external surface with an elevated ridge (Fig. 3Cb), flagellum with long apical setae (Fig. 3Cc), epipodite long, distal margin rounded (Fig. 3Cd). Mxp3 ischiomerus fused without suture line, width-length ratio= 0.7, external margin convex with setae, internal margin with a medial conspicuous projection (Fig. 3Da; white arrow); carpus subconial, external margin with short setae; propodus subconical (Fig. 3Dc); dactylus subspatuliform, wider distally (Fig. 3D; black arrowhead), slightly overreaching propodus, external surface with short plumose setae, external margin with long plumose setae; exopod 1-article, external margin and external surface with short simple setae, flagellum slender, with plumose long setae (Fig. 3E). Sternal third plate with anterior margin sinuous, anterolateral angles with crenu-denticulated margin (Fig. 2C; black arrow), surface scarcely pilose (Fig. 2C; dots); fourth plate slightly globose, surface with microscopic pits (Fig. 2C; hollow circles), distal external angle curved outward, margin crenu-denticulated (Fig. 2C; white arrow). Chelipeds subequals (Figs. 1A–C); merus external surface and carpus anterior margin with plumose setae; chelae width and length subequal, ventral margin microscopically granulated (Fig. 3F, 6C;

コメントの追加 **[TN11]:** Always 12?

388 dashed arrow), dorsal margin slightly cristate and bent inwards; fingers wider than long, length
389 equal, spoon-tipped, tip acute (Fig. 3F), interdactylar gap narrow (vg. Fig. 6C); movable finger
390 shorter than fixed finger, crossed inward when the pincer is closed (vg. Fig. 6C), cutting edge
391 sinuous, with three medial teeth (Fig. 3F; bold arrow) and a mid-distal convex projection (Fig.
392 3F; white dashed arrow); fixed finger cutting edge with nine teeth, faint lamella over the smooth
393 portion of the cutting edge (Fig. 3F; doted arrow), ventral inner surface with short setae. Walking
394 legs similar, relative length W3>W2>W1>W4, segments short, robust, compressed, dorsal
395 margin cristate, ventral surface with plumose setae; merus dorsal margin on W1, W3, W4 with
396 plumose setae, on W2 without setae; dactylus curved, stout, tips acute; W1–W3, dactylus
397 subequal than propodus, of W4 shorter than its propodus (Figs. 1B, 2A). Abdomen symmetrical,
398 subtriangular, six free somites plus a telson, margin with short setae, lateral margin from
399 segments 4–6 slightly concave and narrowing, telson subrounded (Figs. 4B). First gonopod
400 slender, margins sinuous, mid-distal portion notably curved outwards, surface with abundant
401 plumose setae (Fig. 4D). Second gonopod small, flagellum curved outwards, slightly bent
402 inwards, tip pointing upwards, margins convex with a shallow notch (Fig. 2F; black arrow).
403 **Color in life:** Body beige or creamy white, dorsal surface of carapace and chelipeds carpus, and
404 on the external surface of the chelae with red patches. In fixed and preserved specimens this
405 pattern of color remains or it could change from red to light or dark brown (Fig. 1).
406 **Habitat:** Marine. Associated with the sea cucumber *Holothuria* (*Halodeima*) *inornata*, living in
407 its coelom and inside its intestine (Fig. 1G). This holothurian inhabits rocky-sand bottoms in
408 shallow waters (0–18 m).
409 **Variation:** The revised material showed three general outlines on the carapace shape. Between
410 males, a transversally subrectangular carapace shape was observed in 33% (three specimens) of
411 the revised material and comes from Sinaloa, a subovate shape was observed in 56% (five

コメントの追加 **[TN12]:** What is relationships between these outlines and size??

412 specimens) of the material and comes from Guerrero and Oaxaca, and a subtrapezoidal shape in
413 11% (one specimen) comes from Oaxaca. In females, the subrectangular shape was observed in
414 11% (five specimens) of the material and comes from Sinaloa, the subovate shape in 85% (40
415 specimens) and comes from Guerrero and Oaxaca, and the subtrapezoidal shape in 2% (two
416 specimen) and comes from Oaxaca.
417 The subrectangular shape (Figs. 4A, 5A) is defined by a straight and notably projected margin of
418 the frontal lobes, a straight anterior margin in which the hepatic notch in males is notably deeper,
419 eroded, and extended over the carapace (Fig 4A, white arrow) but in females is less conspicuous
420 (Fig. 5A, black arrow), and in the male by a truncated and scarcely projected lateral lobes in
421 which the anterior portion in the male is concave (Fig. 4A, black arrow) but in the female is
422 straight. Instead, the subovate shape (Figs. 4E, I, 5D, G) is defined by an entire even margin
423 which is outlined by the slightly oblique and scarcely projected frontal lobes, the convex
424 anterolateral margin continues smoothly to the lateral margin forming a notably convex lobe
425 (Figs. 4E, I, black arrows) in which the hepatic notch in the males is shallow, slightly eroded and
426 less extended over the carapace (Fig. 4E, I, 5 D, G, white arrows). Meanwhile, the subtrapezoidal
427 shape is defined by the scarcely projected margin of the frontal lobes, which continues evenly
428 and smoothly to the straight anterolateral margin forming notably projected lateral lobes (Fig.
429 1D, 6A). In all the females, the margin of the frontal lobes and the eyes are not visible in dorsal
430 view and only a slight notch is visible (Figs. 5A, D, G, white arrows), because the frontal-dorsal
431 surface is more convex than in males, but if the carapace is placed so that the posterior margin
432 line of the carapace cannot be seen, then the general carapace outline looks like the males from
433 Guerrero or Oaxaca (v.g. Figs. 4E, I). Also, in frontal view, the convexity of the frontal-dorsal
434 surface allows a pair of inflated and only drawn lobes on the surface to be seen. The remarkably
435 convex frontal-dorsal surface which obscures the frontal margin and the eyes in dorsal view, was

observed in 16 specimens (15 females, one male), and the less convex shape was observed in 39 specimens (31 females, eight males). This notably convex shape was more frequent in ovigerous females (10 specimens, 67%) than in non-ovigerous ones (five specimens, 33%). Despite the variation in the shape of the carapace in both sexes, in all cases the CW/CL ratio is the same; plus, the length measured from the notch of the margin of the frontal lobes to the external orbital angle, and that of the external orbital angle to the posterior angle of the hepatic notch, are the same.

Regarding the Mxp3, the ischiomerus external margin appears notably convex on its mid-distal portion or slightly even thoroughout its length, and its inner margin could have a concave or sinuous mid-distal portion; even so, the inner margin always has a blunt or slightly acute projection (Figs. 4 Ca, Ga, Ka, 5Ca, Fa, Ia; black arrow), but its width/length ratio is constant in all the outlines' variations. The carpus is conical, the main appearance variation is its length and the convexity or straightness of its dorsal margin, but that is only related with the drawing perspective (Figs. 4Cb, Gb, Kb, 5Cb, Fb, Ib), yet in all cases there is a projected ridge on the internal surface which has a conspicuous tuff of setae. The propodus also looks variable in its width/length ratio and in its more acute or rounded distal margin, nevertheless that is the result of the way in which the piece was mounted; despite that, its proximal ventral margin always forms a straight angle in which the dactylus is jointed (Figs. 4Cc, Gc, Kc, 5Cc, Fc, Ic). Finally, the dactylus shows two closely related outlines, one subspatulated and the other suboblong, the first has a more expanded distal portion instead of a narrow shape as in the latter; nevertheless, its distal margin always slightly overreaches the propodus (Figs. 4Cd, Gd, Kd, 5Cd, Fd, Id).

Variation in the ornamentation of the chelae fingers is observed. Between males, the cutting edge of the movable finger has two to three proximal blunt or acute teeth (Figs. 4B, F, J, black arrows), the medial tooth is simple (Figs. 4B, J, white arrow) or bicuspid (Fig. F, white arrow),

and the subdistal projection is acute (Figs. 4B, F, white dashed arrow) or blunt (Fig. 4J, white dashed arrow); the fixed finger has six to nine blunt (Fig. 5B) or acute (Fig. 5F, J) teeth, and the middle or more conspicuous tooth is always bicuspid (Figs. 4B, F, J, black arrow). Between females, the movable finger shows two to three acute teeth (Fig. 5B, E, H, black arrow), an acute (Figs, 5B, E, white arrow) or blunt (Fig. 5H, white arrow) medial tooth, and a blunt subdistal projection (Figs. 5B, E, H, white dashed arrow); the fixed finger has four to thirteen teeth with a bicuspid blunt medial tooth (Fig. B, E, black arrow) or a simple acute one (Fig. H, black arrow). Only one specimen (DECA-1172) has different size chelae and a different teething pattern on the cutting edge of the fixed finger (Fig. 5J, K).

The first gonopod of the males shows variation in the degree of curvature and the amount of the distal portion that is curved, and also in the general outline shape of the gonopod tip, but may be similar in different stages of development. In this sense, the general appearance in the abdominal view, of males from Sinaloa and Oaxaca is more similar because the external and internal margins are sinuous (Figs. 4D, L), the curvature degree is approximately 90° (Fig. 4D) and 75° (Fig. 4L) respectively, the tip of the external margin is truncated (Figs. 4De-f, Le-f; white arrow), and the ventral margin of the tip has a blunt projection (Figs. 4De, Le; black arrow); while in that of Guerrero, the external and internal margins are les sinuous and the curvature degree is approximately 65°(Fig. 4H), the tip of the external margin is convex (Fig. 4He-f; white arrow), and the ventral margin of the tip has a pointed projection (Fig. 4He; black arrow). Also, in sternal view, the ventral process shape of the internal margin tip is variable, in males from Sinaloa it is obtuse (Fig. 4D-f; black arrow), while in those from Guerrero it is convex (Fig. 4H-f; black arrow) and those from Oaxaca is oblique (Fig. 4L-f; black arrow), but this is variable also between the sizes of the crabs.

Remarks: All the biological material examined shows phenotypic variation, particularly between the individuals from the type locality in Mazatlan with respect to those of Guerrero and Oaxaca, but molecular evidence show no differentiation. Now with the description of the male morphology it is possible to differentiate *Holothuriophilus trapeziformis* from *H. pacificus* with certainty because the carapace could be subrectangular (Fig. 4A, 5A), suboval (Fig. 4E, I, 5D, G) or subtrapezoidal (Fig. 1A, D, 2A, 6A) in the former but it is subcuadrangular in the latter (Fig. 6E). *H. trapeziformis* has the Mxp3 dactylus with its distal portion notably expanded, the external distal margin slightly truncated, and the flagellum of the exopod is long and robust (Figs. 6B, J, K, 7A); but in *H. pacificus* the distal margin is rounded and the flagellum of the exopod is long and slender (Figs. 6F, 7D). The first gonopod of *H. trapeziformis* has a more sinuous lateral margins with a larger distal portion curved outwards with abundant setae (Fig. 7C); however, in *H. pacificus* it is straight with only the distal portion slightly curved outwards, and with less abundant setae (Fig. 7F). The abdomen of *H. trapeziformis*, in males, is subtriangular with lateral margins narrowing from the fourth to the sixth somite, the third somite has notably convex lateral margins, the sixth somite has notably concave lateral margins, and the telson is subrounded and wider than long (Fig. 7B); yet in *H. pacificus* it is triangular, the lateral margins are almost straight, the third and sixth somite lateral margins are concave, and the telson is subtriangular and longer than wide (Fig. 7E). In the case of *Holothuriophilus trapeziformis* adult ovigerous and non-ovigerous females, the abdomen is suboval and wider than long, the first somite has convex lateral margins, the second somite has a sinuous distal margins, the third somite has an oblique and downward lateral margins, the sixth somite has oblique and outward lateral margins, and the telson has a length to width ratio ca. 0.2 (Fig. 6D); instead in *H. pacificus* it is suboval and longer than wide, the first somite has concave lateral margins, the second somite has an almost straight

distal margins, the third somite has oblique and upwards lateral margins, the sixth somite has convex lateral margins, and the telson has a length to width ratio ca. 0.3 (Fig. 6H).

Distribution and ecological comments: The present study allows us to increase the previous known distribution range from Punta Tiburon, Sinaloa to playa Las Gatas, Guerrero, to the south, 615 km to playa Tejon, Oaxaca. We found crabs in the coelom cavity and near the cloaca of the host, as mentioned by Manning (1993), Campos, Peláez-Zárate & Solís-Marín (2012) and Honey-Escandón & Solís-Marín (2018) and, for the first time, it is registered within the intestine (Fig. 1G).

Holothuria (*Halodeima*) *inornata* is distributed throughout the Tropical Eastern Pacific from the Gulf of California, Mexico to Ecuador, and in the temperate island Lobos de Afuera, Peru (Prieto-Rios et al. 2014; Honey-Escandón & Solís-Marín 2018). It also, represents an important fishery resource throughout its distribution range (Santos-Beltrán & Salazar-Silva 2011), yet there are no records for *Holothuriophilus trapeziformis* outside the Pacific coast of Mexico.

Molecular approach

DNA Barcodes

From the 56 examined crabs (Table S1), 51 were processed. The number of base pairs was between 549 bp and 648 bp for 37 specimens with a sole Barcode Index Number (BIN; Ratnasingham & Hebert 2013) in the BOLD database: ADE9974. Of those, 35 produced a high-quality barcode. The 14 crabs that could not be amplified correspond to old museum material and to recent collections that are not fixed according to the Elías-Gutiérrez et al. (2018) protocol. A BLAST query in GenBank confirmed our sequences to belong to a brachyuran lineage. Finally, in the case of the hosts, none could be amplified.

Phylogeny and distance analysis

The best nucleotide substitution model according to AIC and BIC criterion was General Time Reversible under a gamma distribution (GTR+G) model (Nei & Kumar 2000). The Maximum-Likelihood (ML) distance method under the selected model delimited the 37 sequences of *Holothuriophilus trapeziformis* from the dataset (DS-PINMX1HT) in a single cluster; however, two sub-groups are defined, one for Sinaloa (northern) and the other for Guerrero and Oaxaca (southern). These two clusters are well separated from the sister group, *H. pacificus*, in the maximum likelihood tree (ML) as can be seen in figure 8, with a 12 to 14% of divergence among all specimens. *Holothuriophilus* is also related to the *Calyptraeotheres* clade, but far from other species (Fig. 8) with an interspecific divergence ranging from 12 to 19%.

Intraspecific DNA polymorphisms and historical demography

Although the *Holothuriophilus trapeziformis* clade shows two well differentiated groups, its intraspecific divergences ranged from 0 to 2.2% This is congruent with the BOLD distance summary analyses which show an average distance of 0.73% and a maximum of 2.27% for sequences with more than 500bp. For *Holothuriophilus trapeziformis* from the Pacific coast of Mexico we identified 34 nucleotide substitutions (28 transitions, 6 transversions), and 33 polymorphic sites (14 parsimony informative sites and 19 singleton variables) that defined 22 unique COI haplotypes with a moderate mean number of nucleotide differences between pairs (k= 3.775), and total genetic diversity estimations indicate a high haplotype diversity (*Hd* = 0.914) but a moderated nucleotide diversity (π= 0.007) (Table 1). Within-regions the haplotype diversity was high in all localities (ranging from 0.874–0.964), and the nucleotide diversity shows an increment along the considered latitudinal gradient from south to north (Oaxaca and Guerrero with 0.003 and 0.006, respectively, and Sinaloa with 0.009) (Table 1).

Of the 22 haplotypes (Table 2), the H3 is the most abundant and is present in all sites. Nevertheless, two haplogroups were well defined (Fig. 9); one haplogroup is formed by 18 haplotype related to Guerrero and Oaxaca localities from which H3 is most frequent, and the other haplogroup is represented by four exclusive haplotypes from Sinaloa (H1, H2, H4, H5).

A genetic differentiation among sample sites was demonstrated by pairwise Φ_{ST} values. A low value was observed between Guerrero vs. Oaxaca (Φ_{ST}= 0.06286, p= 0.027), while a high value was shown between Sinaloa vs. Guerrero (Φ_{ST}= 0.44434, p= 0.004) and Sinaloa vs. Oaxaca (Φ_{ST}= 0.57864, p= ≤ 0.001).

SAMOVA results indicated that genetic differentiation was better when considering two groups (k = 2; Group 1: Sinaloa, Group 2: Guerrero-Oaxaca) because 53% of the variance is explained (Table 3), in contrast to 44% of the variation when considering a single group (k = 1; Group 1: Sinaloa-Guerrero-Oaxaca) (Table 4). This result confirmed the groups previously defined by the haplotype network as haplogroup A (Guerrero-Oaxaca) and haplogroup B (Sinaloa) with a Φ_{CT}= 0.53 (p≤0.001) as shown in table 3. The Mantel test showed significant relationships among these variables (r=0.604; p < 0.0001) suggesting patterns of isolation by distance.

Under the coalescent method, the overall *Holothuriophilus trapeziformis* mismatch distribution indicates a significant ragged unimodal distribution (Fig. 10A; r = 0.07780, p = 0.0280; R2 = 0.11407, p = 0.0000). At the regional scale, in Sinaloa a multimodal distribution was observed (Fig. 10B; r = 0.22104, p = 0.57700; R2 = 0.20112, p = 0.0260), whereas in Guerrero it was bimodal (Fig. 10C; r = 0.15286, p = 0.200800, R2 = 0.18034, p = 0.0260), and in Oaxaca it was unimodal (Fig. 10D; r = 0.08442, p = 0.23400; R2 = 0.12867, p = 0.00600).

The neutrality test of Tajima's D for the overall *H. trapeziformis* was negative and significant (Tajima's D = -1.83464, p = 0.01100), pointing to a population expansion. When Fu's (Fs) is taken into account, all the population levels were negative and significant, also indicating an

expansion. Finally, the raggedness index (r) indicates a population growth, as its values were low but not significant in all the population levels, as well as the R2 that indicated an expansion model in all cases (Table 1). We preferred to use the Tajima's D and the R_2 estimations, instead of Fu's (Fs), because of the small sample size, and because it is known that these parameters are particularly recommended when recombination levels are unknown (Ramos-Onsins & Rozas, 2002; Ramírez-Soriano et al., 2008)

Discussion

We detect high variability in some of the most external features in *Holothuriophilus trapeziformis*. The general body appearance of the northern specimens from the type locality (Mazatlan, Sinaloa) is more robust and eroded with shorter pereiopod segments than those of the southern localities (Guerrero and Oaxaca). Taking into account that for pinnotherids taxonomy a crucial goal is to provide a complete description with detailed illustrations of common and unusual structures (Derby & Antema 1980; Ahyong, Komai & Watanabe 2012; Salgado-Barragan 2015) for comparative purposes, then the selected material in this research is 2 mm less than the female described by Bürger (1895*)* and 0.3 mm greater than the female lectotype described by Ahyong & Ng (2007*)*. Therefore, the morphological variation of the females could be contrasted with the available illustrations, and the description of the species was completed with the morphology of the male. In the available female illustrations, a presumable specimen from the type locality shows a subrounded carapace shape (Bürger, 1895: 380–381, pl. 9, fig. 26, plate 10, fig. 25; CW = 14 mm, CL = 10 mm) and the lectotype, also from the type locality, shows it as subovate (Ahyong & Ng 2007: 214, Fig. 20A; CW = 7.7 mm, CL = 4.8 mm; in the present document see Fig. 6I), but another from Guerrero has a subtrapezoidal shape (Campos, Peláez-Zárate & Solís-Marín 2012: 60, fig. 2B; CW = 9.1 mm, CL = 5.2 mm). In the revised females, variations of the carapace shape are due to the projection of the lateral lobes and by the

convexity of the front-dorsal surface; the revised males in contrast with the females, have less expanded and projected lateral lobes, a more pilose pterigostomian region, and slightly less abundant setae on the pereiopods. Additionally, the first gonopod of the males shows a different appearance in the three geographic regions, but that from Sinaloa is more similar to that of Oaxaca in its general shape, setae pattern, and degree of curvature of the apical portion, than to that from Guerrero (see Fig. 4D, H, L) when the most developed stage is considered; however, that can be variable within the same locality. In contrast, the second gonopod (Fig. 2F) shows no differences between all the examined males. Variations in the Mxp3 between sexes is common, but setae pattern and abundance correspond to that shown by Campos, Peláez-Zárate & Solís-Marín (2012; Fig. 6K). We believe that the state of development and the position in which the specimen was observed and drawn are the primary causes of the differences between the available illustrations.

Despite the facts mentioned above, we can conclude that *Holothuriophilus trapeziformis* is different from *H. pacificus* not only by the absence of a space when the fingers are closed (see Figs. 6C, G) as pointed out by Campos, Peláez-Zárate & Solís-Marín (2012), but also because *H. pacificus* does not have a convex mid-distal projection on the cutting edge of the mobile finger (Fig. G) as *H. trapeziformis* does (Fig. 4B, F, J, 5B, E, H; dotted arrow). Additionally, these species can be separated by the shape of the abdomen of both sexes (Figs. 6D, H, 7B, E), and by the structure of male's first gonopod (Figs. 7C, F). Also, *H. trapeziformis* has a granulated ventral surface on the palm of the chelae, mostly on larger sized mature crabs (Fig. 7 C; dashed arrow); that condition is not documented for *H. pacificus* in Garth (1957; fig. 7G) and its synonyms (=*Leucosia pacifica* Poeppig, 1983 =*Pinnaxodes silvestrii* (Nobili, 1901) =*Pinnaxodes meinerti* Rathbun, 1904).

Regarding the molecular approach, *Holothuriophilus trapeziformis* did not have previous genetic information. The success of the COI gene amplification of *Holothuriophilus trapeziformis* was accomplished after the implementation of the chilled ethanol preservation protocol suggested by Elías-Gutiérrez et al. (2018). Due to the thickness of the cuticle, we decide the injection of ethanol inside the body of the crabs through the joints of the armature, as well as the use of zooplankton primers (Prosser, Martínez-Arce & Elías-Gutíerrez 2013) instead of Folmer or other generic primers. With these improvements, we obtained the amplification of 72% of the total sample and a total of 69% sequencing success on a group that is considered difficult to work with COI genes (Mantellato et al. 2016) and this allowed to us to obtain some basic genetic parameters (Table 8). Those results allowed us to confirm the taxonomic status of *Holothuriophilus trapeziformis* as a valid species since the different analyses based on the COI gene fragment (vg. Barcode BIN, IDtree, and Maximum likelihood phylogenetic topology) indicated a divergence ranging from 12 to 14% against *H. pacificus*. These values are above the 3% threshold proposed by Hebert et al. (2003) as a tool to recognize taxonomic units. They also fall into the pairwise distance ranges proposed for crustacean congeners (1.5% to 3.3%, average= 2.5%; Lefébure et al. 2006) and Decapoda congeners (4.92% to 31.39 %, average= 17.16%; Costa et al. 2007), although these values are slightly lower, they fit within the ranges for pinnotherids (15.5% to 24.6%, average= 18.3%; Ocampo et al. 2013). Our Maximum-Likelihood tree agrees with that of Palacios-Theil, Cuesta & Felder (2016) in regards with the association of the genus *Holothuriophilus* and *Calyptraeotheres*.

Phenotype variation is a result of a plastic response to different environmental pressures, particularly when the species shows a wide distribution in heterogeneous or geographically isolated environments (Hurtado, Mateos & Santamaria 2010; Rossi & Mantelatto 2013); also, recent or historical processes that limit the flow of genes determine a genetic structure in the

populations of the species, but it has been considered that in the marine province the species exhibit low levels of differentiation even if there are environmental barriers (Wares, Gaines & Cunningham 2001; Avise 2009). However, in brachyuran crabs, much evidence has been argued against that, principally because of the particularities of the geographic areas, the habitat peculiarities, and by the species life history as has been documented for grapsids (Cassone & Boulding 2006), ocypodids (Laurenzano, Mantelato & Schubart 2013), pinnotherids (Ocampo et al. 2013), sesarmids (Zhou et al. 2015), and varunids (Zhang et al. 2017).

Pinnotherid crabs are known to have a complex life cycle and ample time for their development lasting from 26 to 30 days (Bousquette 1980; Hamel, Ng & Mercier 1999; Ocampo et al. 2011), which allows them to maintain connectivity between populations throughout their geographical distribution range (Ocampo et al. 2013); however, in this case, connectivity through larval dispersal may be more restricted due to their symbiotic behavior and the specificity of the relationship with their host than due to other environmental factors (Haines, Edmunds & Pewsey 1994; Hamel, Ng & Mercier 1999; Ocampo et al. 2012, 2013; Guilherme, Brustolin & de Bueno 2015; Becker & Türkay 2017*)*. *Holothuriophilus trapeziformis* has been considered as an endobiontic parasite of their host since its description (Nauck 1880), but no other related reference confirms that type of interaction (Bürger 1895; De Man 1887; Ng & Manning 2003; Ahyong & Ng 2007; Campos, Peláez-Zárate & Solís-Marín 2012). Of the thirteen pinnotherids known to develop this endobiotic way of life, living near the respiratory trees, the coelomic cavity, or the posterior part of the digestive gut, and could or could not cause detrimental effects (Hamel, Ng & Mercier 1999; Ng & Manning 2003); only the life cycle of *Holotheres haling* has been described in detail (Hamel, Ng & Mercier, 1999). In contrast, the life cycle of *Holothuriophilus trapeziformis* is still unknown. As a starting point to generate information about it, we only collected the holothurian species *Holothuria* (*Halodeima*) *inornata* in accordance to

Honey-Escandón & Solís-Marín (2018), but some other holothurians from the South Pacific coast of were also examined in search of the symbiont, with no success. Thus, we found the crab inhabiting in a membranaceous cyst through the coelomic cavity and inside of the gut, and never found more than one crab together.

Besides the cyst cavity wound produced by the crab on the cloacal internal wall, we also found some crabs with pieces of the respiratory tree in their pincers and inside their buccal cavity. *Holothuriophilus trapeziformis* has spoon-tipped fingers but not much is known about the precise function of this kind of condition, but it is associated with feeding on detritus, scooping up mucus from corals, picking up soft foods, scraping off encrusting algae, effective gripping of filamentous algae, or scraping epilithic algae off coral rock (Davie, Guinot & Ng 2015); however, there is no information on this respect for pinnotherids with this condition. It is necessary to examine the stomach contents to corroborate that the crab feeds only on the host tissue or also on detritus of the intestine, and to evaluate in some way the physiological damage produced in the host to determine with certainty if the agonistic interaction corresponds to a parasitism or a commensalism.

With this context, the *Holothuriophilus trapeziformis* morphological variation and significant genetic differentiation through its distribution range was indicated and supported by the haplotype network, the Φ_{ST} index, SAMOVA and Mantel test, mainly in the distinction between the northern and southern forms. But, the range of the intraspecific distance values corresponds to the thresholds proposed for crustaceans (Lefébure et al. 2006), for decapods (Costa et al. 2007), and for pinnotherids (Ocampo et al. 2013) to maintain the intraspecific delimitation. In addition, a sudden expansion of population growth was evidenced by a gene flow from the south to the north, due to the overall high haplotype and low nucleotide diversity detected with 22 haplotypes in 37 individuals from which all the haplotypes derived from a possible ancestral haplotype (H3)

694 in Oaxaca. The overall unimodal mismatch distribution displayed, and the negative and
695 significant results of the neutrality Tajima's (D) and Fu's (Fs) test, support that scenario.
696 Nevertheless, the morphological difference and the pairwise genetic distance observed in Sinaloa
697 could represent a process of differentiation since the mismatch distribution there shows a
698 multimodal form that is statistically not significant (Fig. 10B; r = 0.22104, p = 0.57700; R2 =
699 0.20112, p = 0.0260). Sample size is significant and correlated with the number of haplotypes
700 (Cassone & Boulding 2006) and probably has an effect over the mismatch distributions.
701 Additionally, in this northern area the presence of the Thermocline Cabo Corrientes Dome
702 (Gómez-Valdivia, Parés-Sierra & Flores-Morales 2015) probably acts as a physical barrier to the
703 flow of genes from the south. Northern specimens are genetically and morphologically different
704 to those of the southern Mexico, but correspond with the model of isolation by distance, in which
705 the differences between populations are due to limited gene flow because of a restricted
706 geographical dispersion, the near-surface marine circulation patterns, the discontinuity of
707 habitats, and the frequency of sexual reproduction. This kind of situation has been demonstrated
708 for other decapods with a complex life cycle (Rossi & Mantelatto 2013).
709 Considering the *Holothuriophilus trapeziformis'* way of life and its relationship with its host, the
710 environmental pressures determining the genetic connectivity correspond to geographical barriers
711 (extended estuarine areas and wide sandy beaches between the rocky shores) and oceanographic
712 processes (Mexican Coastal Current, Thermocline of Cabo Corrientes Dome, and Thermocline of
713 the Tehuantepec Bowl) through the Pacific coast of Mexico (Hurtado et al. 2007; Paz-García et
714 al. 2012; Gómez-Valdivia, Parés-Sierra & Flores-Morales 2015), which influence the gene flow.
715 This is important because the host, *Holothuria* (*Halodeima*) *inornata*, shows a wide distribution
716 range across the subtropical American Pacific coast, with two well defined genetic populations:
717 A Mexican one—Gulf of California to Oaxaca— and a Panamic one—from Chiapas, Mexico to

718 Peru, inhabiting rocky shores (Prieto-Rios et al. 2014). Despite the specificity of the association
719 with *Holothuria* (*H.*) *inornata* (Honey-Escandón & Solís-Marín 2018) no records of
720 *Holothuriophilus trapeziformis* south to Oaxaca are known. Until now, and with all the evidence
721 presented here, *Holothuriophilus trapeziformis* has the status of endemic species of Mexico;
722 therefore, in order to establish that status, it is necessary to confirm the presence or absence of the
723 species in the distribution range of *Holothuria* (*H.*) *inornata* outside of Mexico.
724 We can conclude that the taxonomic status of *Holothuriophilus trapeziformis* is now completed,
725 recognized based on the morphology of both sexes, and the genetic and demographic historical
726 analyses, that confirm the taxonomic status of all the revised material as the same species by
727 linking all the sequenced material with a new DNA barcode (BOLD:ADE9974) different from
728 that of *H. pacificus* (BIN, BOLD: ABV9743; boldsystems.org). We also suggest that
729 morphological plasticity is the result of an isolation by distance experienced by the individuals in
730 the considered regions after a sudden population expansion throughout their life history. In spite
731 of that, however, the specialized relationship with their host, the restricted habitat in which they
732 live, and local environmental barriers are perhaps the main forces that have caused this plasticity.
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