

Reinstatement of *Phascolosoma* (*Phascolosoma*) *varians* Keferstein, 1865 (Sipuncula: Phascolosomatidae) based on morphological and molecular data

Itzahi Silva-Morales ^{Corresp. 1}

¹ Departamento de Sistemática y Ecología Acuática, El Colegio de la Frontera Sur, Chetumal, Quintana Roo, Mexico

Corresponding Author: Itzahi Silva-Morales

Email address: itzahi.silva@estudianteposgrado.ecosur.mx

Phascolosoma (*P.*) *varians*, a sipunculan species known from the Greater Caribbean, was designated as a synonym of *Phascolosoma* (*P.*) *nigrescens*, which was originally described from Fiji. Their synonymy was primarily based upon an interpretation that these two species were morphologically indistinguishable. After its designation as a synonym, no further detailed analyses of morphological or molecular characteristics were performed to corroborate the assumed widespread distribution of *Phascolosoma* (*P.*) *nigrescens*. In this study, *Phascolosoma* (*P.*) *varians* is redescribed, and notable differences between this species and its proposed senior synonym are presented. These two species differ in the shape of their hooks, the spatial attachment of nephridia to the body wall, and the morphology of the contractile vessel. Additionally, there is high genetic divergence between nucleotide sequences within their respective cytochrome c oxidase subunit 1 (COI) genes, which supports the morphological data. Herein, the synonymy of *Phascolosoma* (*P.*) *varians* with *Phascolosoma* (*P.*) *nigrescens* is rejected due to morphological and molecular differences. Furthermore, the assumed widespread distribution of *Phascolosoma* (*P.*) *nigrescens* is still considered as questionable.

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Itzahí Silva-Morales¹

¹Departamento de Sistemática y Ecología Acuática, El Colegio de la Frontera Sur, Chetumal, Quintana Roo

Corresponding Author:

Itzahí Silva-Morales¹

¹Av. Centenario, Chetumal, Quintana Roo, 77014, Mexico

Email address: itzahi.silva@estudianteposgrado.ecosur.mx

Abstract

Phascolosoma (*P.*) *varians*, a sipunculan species known from the Greater Caribbean, was designated as a synonym of *Phascolosoma* (*P.*) *nigrescens*, which was originally described from Fiji. Their synonymy was primarily based upon an interpretation that these two species were morphologically indistinguishable. After its designation as a synonym, no further detailed analyses of morphological or molecular characteristics were performed to corroborate the assumed widespread distribution of *Phascolosoma* (*P.*) *nigrescens*. In this study, *Phascolosoma* (*P.*) *varians* is redescribed, and notable differences between this species and its proposed senior synonym are presented. These two species differ in the shape of their hooks, the spatial attachment of nephridia to the body wall, and the morphology of the contractile vessel. Additionally, there is a high genetic divergence between nucleotide sequences within their respective cytochrome c oxidase subunit 1 (COI) genes, which supports the morphological data. Herein, the synonymy of *Phascolosoma* (*P.*) *varians* with *Phascolosoma* (*P.*) *nigrescens* is rejected due to morphological and molecular differences. Furthermore, the assumed widespread distribution of *Phascolosoma* (*P.*) *nigrescens* is still considered as questionable.

Introduction

The phylum Sipuncula comprised of 320 species as recorded by Stephen & Edmonds (1972), but after numerous revisions over a period of approximately twenty years by Edward and Norma Cutler, the total number was reduced to 149 valid species (Cutler, 1994). The reduced number of sipunculan taxa may call into question previous records, lead to possible taxonomic errors among field investigators, and suggest that most, if not all, species have been accounted for. However, after 1994, 13 new species have been described (Kawauchi & Rice, 2009; Hylleberg, 2013; Saiz *et al.*, 2015; Silva-Morales *et al.*, 2019). Importantly, in the Greater Caribbean region alone, 38 valid species have been recorded, of which 40% correspond to species with type localities outside of the Greater Caribbean, with 7 of those species belonging to Phascolosomatidae (Quiroz-Ruiz & Londoño-Mesa, 2015).

Reduction in the number of species by Cutler (1994) was performed by proposing extensive lists of synonyms, and when utilized by subsequent investigators, those lists have most likely led to a number of incorrect species identifications. For example, in the Greater Caribbean, *P. (P.) nigrescens* has been reported by Cutler & Schulze (2004) from Barbados, Schulze & Rice (2004) from Belize, Schulze (2005) from Panama, and Frontana-Urbe *et al.* (2018) from the Mexican Caribbean. These records may reflect the fact that Cutler (1994) considered the distribution of *P. (P.) nigrescens* as widespread and circumtropical, found generally between 30° N and 30° S in shallow waters of the Indian, Pacific, and Atlantic oceans.

The extensive synonymization approach by Cutler was proposed in part by an assumption that many sipunculan species have wide geographic distributions. Those with wide distributions were thought to be possible due to the high dispersal capability of species with teleplanic pelagosphera larvae, which were inferred to remain in the water column for up to six months base upon laboratory experiments (Rice, 1976). *Phascolosoma* (*Phascolosoma*) *varians* has a

Category 4 developmental pattern (Rice, 1970): Indirect development with two pelagic larval stages, trochophore and planktotrophic pelagosphaera. Planktotrophic pelagosphaera larvae can be either short-lived (weeks) forms or larger long-lived (months) teleplanic larvae, as in *P. (P.) varians* (Boyle & Rice, 2014). The teleplanic larvae of the Phascolosomatidae are characterized by having cuticular papillae (Scheltema & Rice 1990).

However, recent molecular analyses revealed potential taxonomic problems at the species level, where small morphological differences also were shown to correspond with distinct species, thus rejecting previously assumed wide distributions for some of those species (Staton & Rice, 1999; Kawauchi & Giribet, 2010; Schulze *et al.*, 2012; Kawauchi & Giribet, 2014; Johnson *et al.*, 2016; Silva-Morales *et al.*, 2019). Because of this, Kawauchi, Sharma & Giribet (2012) proposed to clarify the taxonomic status of each species through a meticulous case-by-case analysis, integrating molecular data while considering that some species names that are currently hidden under synonyms deserve to be restored. In regards to molecular data, the mitochondrial cytochrome oxidase c subunit I (COI) an efficient identification tool for metazoan species, making it the core fragment for DNA barcoding (Hebert, Ratnasingham & de Waard, 2003).

Keferstein (1865) described *Phascolosoma (Phascolosoma) varians* from St. Thomas, West Indies, and *P. (P.) nigrescens* from Fiji. Cutler & Cutler (1983; 1990) reviewed the subgenus *Phascolosoma (Phascolosoma)*, and redesignated 13 species, previously described and recorded from all around the world, as synonyms of *P. (P.) nigrescens*. The identification key by Cutler (1994) indicates that the diagnostic characters of *P. (P.) nigrescens* include the following: *a distinct clear streak in particular hooks with observable swelling in the middle of vertical and horizontal portions*. *Phascolosoma (P.) varians* was one of multiple species included as a junior synonym of *P. (P.) nigrescens*.

Herein, a detailed redescription of *P. (P.) varians* based upon topotypic specimens and additional material from other Caribbean localities is provided. Furthermore, *P. (P.) varians* is reinstated due to both morphological and molecular differences that distinguish it from its proposed senior synonym, *P. (P.) nigrescens*.

Materials & Methods

Specimens from the collections of the Marine Invertebrate Museum (UMML), Rosenstiel School of Marine and Atmospheric Science, University of Miami; Invertebrate Collections of the Florida Museum of Natural History (UF), University of Florida; and the Reference Collection of Benthos (ECOSUR) of El Colegio de la Frontera Sur, Chetumal, Mexico were reviewed.

Redescription of the species was primarily based upon a topotypic specimen, but additional materials from other Caribbean localities were also assessed for species-specific variations. Standardized descriptions included external and internal anatomy. The descriptions of hooks and papillae followed the terminology proposed by Cutler (1994). To measure the angle between the primary tooth and the hook, a line *X* was drawn perpendicular to the base through the most anterior part of the concave side, and a line *Y* was drawn from the tip until intersect *X* in the middle of the point (see Cutler 1994:161-162, fig. 44A).

Hooks and papillae were extracted with fine forceps for examination under an Olympus CH30 compound light microscope. Hooks were excised from three different regions (proximal, median and distal) of the ringed area of the introvert. Papillae were described from three different regions (anterior, median and posterior) of the trunk, and also from the distal introvert. Furthermore, these structures were examined using SEM to achieve a more detailed examination. For SEM preparation, the complete introvert was dehydrated through a series of increasing concentrations of hexamethyldisilazane (HMDS). Once air-dried, the introvert was mounted on an aluminum stub and coated with gold for observation with a JEOL JSM-6010Plus-LA scanning electron microscope at the Scanning Electron Microscopy Laboratory (LMEB), ECOSUR-Chetumal. Digital photographs of selected internal and external features were obtained with a Canon X6 digital camera mounted on a Leica MZ75 dissecting stereomicroscope. All images were rendered from a series of optical focal planes with HeliconFocus v6.7.1 (HeliconSoft 2007) to improve the depth of field for each specimen or set of specimens that were photographed.

For molecular analyses, eight COI sequences with an alignment length of 541 bp, from specimens identified as *Phascolosoma* (*P.*) *nigrescens* were retrieved from GenBank. One of them from Barbados (DQ300139), two from Florida (DQ300142, AY161122), one from Broome, Australia (DQ300143), two from New Caledonia (JN865121, JN865122), one from Israel (DQ300140), and another from South Africa (DQ300141). Also, a COI sequence from *Phascolosoma* (*P.*) *granulatum* Leuckart, 1828 (DQ300138) and two sequences from *Phascolosoma* (*P.*) *agassizii* Keferstein, 1866 (JQ904338, JQ904337) were included for comparison.

All sequences were aligned using the ClustalW method. Selection of the best model of substitution was determined according to the lowest Bayesian Information Criterion scores (BIC). From the BIC results, the Tamura 3-parameter (Tamura 1992) model with a discrete Gamma distribution (+G) with five categories, assuming a fraction of sites is evolutionarily invariable (+I), was selected to construct a tree by maximum likelihood analysis. The Kimura 2-parameter model (Kimura 1980) was used to estimate the average evolutionary divergence over sequence pairs within and between species. All analyses were carried out with Mega 7 (Kumar, Stecher & Tamura, 2015).

Results

Systematics

Family Phascolosomatidae Stephen & Edmonds, 1972

Phascolosoma Leuckart, 1828

Phascolosoma (*Phascolosoma*) Leuckart, 1828

Type species. *Phascolosoma granulatum* Leuckart, 1828

Diagnosis. Body wall muscles separated into distinct bands. Spindle muscle attached posteriorly; introvert hooks without accessory spinelets (after Cutler 1994).

***Phascolosoma (Phascolosoma) varians* Keferstein, 1865 reinstated**

Phascolosoma varians Keferstein 1865: 424-426, pl. 32, Fig. 22. de Quatrefages 1865: 623.

Wesenberg-Lund 1954: 7-8. Rice & Macintyre 1982: 314.

Phymosoma varians: Selenka 1883: 69-70, pl. 9, figs. 124-127. Shipley 1890: 1-24, fig. pls. 1-4, figs. 1-32.

Physcosoma varians ten Broeke 1925: 85.

Phascolosoma (Phascolosoma) varians: Stephen & Edmonds 1972: 327-328, fig. 39I.

Phascolosoma nigrescens Cutler 1994 (*partim*): 179-181; Cutler & Schulze 2004: 226; Schulze 2005: 526; Frontana-Urbe *et al.* 2018:174, fig. 5a-b; (non Keferstein, 1865).

Material examined

USA, Florida. UMML 26.5, 2 specimens, Bear Cut, Key Biscayne, 25°43'54.33"N, 80°09'26.16"W, May 7, 1961, coll. P. Robertson. UMML s/n, 1 specimen. Margot Fish Shoal, Dade Co, Apr 5, 1966, coll. G. Hendrix, bored in coral rubble. UF 292, 1 specimen, N of St. Petersburg, 28°27'33.12"N, 84°16'18.48"W, 30 m, Mar 13, 2011, colls. G. Paulay, N. Evans, F. Michonneau, C. Thacker, R. Williams, A. Baeza. UF 293, 1 specimen, N of St. Petersburg, 28°27'33.12"N, 84°16'18.48"W, hard ground with sponges, 30 m, Mar 13, 2011, coll. G. Paulay. UF 324, 1 specimen, St. Petersburg, 28°33'24.12"N, 84°16'28.20"W, hard bottom, sponge reef, 27 m, May 24, 2012, coll. J. Slapcinsky. UF 325, 1 specimen, St. Petersburg, 28°39'03.96"N, 84°23'03.84"W, 26-30 m, hard bottom, sponge reef, May 25, 2012, colls. G. Paulay, N. Evans, F. Michonneau. **Mexico, Mexican Caribbean, Isla Contoy.** ECOSUR-S62, 3 specimens, Morro Norte, 21°28'32.79"N, 86°47'30.13"W, coralline rock, 2.5 m, Feb 26, 2008, colls. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S63, 2 specimens. Punta Sur, 21°27'37.10"N, 86°47'04.60"W, coralline rock, 1.5 m, Mar 2, 2001, colls. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S69, 1 specimen, Punta Sur, 21°27'37.10"N, 86°47'04.60"W, coralline rock, 1.5 m, Feb 28, 2001, colls. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S70, 1 specimen, Ixlache reef, 21°26'02.52"N, 86°46'56.16"W, coralline rock, 2 m, Feb 25, 2008, colls. S. Salazar-Vallejo, L. Carrera-Parra. **Cancun, Punta Nizuc.** ECOSUR-S87, 1 specimen, 21°01'41.92"N, 86°46'45.72"W, coralline rock, 2.6 m, Aug 31, 1997, colls. S. Salazar-Vallejo, L. Carrera-Parra, M. Ruiz-Zárate. ECOSUR-S88, 2 specimens, 21°01'17.06"N, 86°46'45.95"W, coralline rock, 4 m, Sep 1, 1997, colls. S. Salazar-Vallejo, L. Carrera-Parra, M. Ruiz-Zárate. ECOSUR-S89, 3 specimens, 21°01'17.06"N, 86°46'45.95"W, coralline rock, 4 m, Sep 1, 1997, colls. S. Salazar-

201 Vallejo, L. Carrera-Parra, M. Ruiz-Zárate. ECOSUR-S90, 2 specimens, 21°01'17.06"N,
 202 86°46'45.95"W, coralline rock, 4 m, Sep 1, 1997, colls. S. Salazar-Vallejo, L. Carrera-Parra, M.
 203 Ruiz-Zárate. **Playa del Carmen**. ECOSUR-S86, 4 specimens, Navega pier, 20°37'12.07"N,
 204 87°04'26.63"W, fouling, 1 m, Aug 23, 2003, coll. M. Tovar-Hernández. **Cozumel**. ECOSUR-
 205 S64, 14 specimens, Playa Azul, 20°32'51.98"N, 86°55'46.45"W, coralline rock, 1 m, Mar 25,
 206 2001, colls. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S65, 1 specimen, in front to
 207 SEDENA, 20°31'00.61"N, 86°56'45.52"W, coralline rock, 1.5 m, Mar 24, 2001, colls. S.
 208 Salazar-Vallejo, M. Londoño-Mesa. **Tulum**. ECOSUR-S66, 3 specimens, Playa Aventuras,
 209 20°21'47.20"N, 87°19'53.10"W, coralline rock, 1.5 m, Feb 28, 1999, colls. S. Salazar-Vallejo, J.
 210 Bastida-Zavala. ECOSUR-S91, 1 specimen, Playa Aventuras, 20°21'47.20"N, 87°19'53.10"W,
 211 coralline rock, 1.5 m, Feb 18, 2001, colls. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S92, 4
 212 specimens, Ana y José beach, 20°09'24.22"N, 87°27'13.74"W, coralline rock, 1 m, Feb 11, 2001,
 213 colls. S. Salazar-Vallejo, J. Bastida-Zavala, J. Ruiz-Ramírez. ECOSUR-S96, 3 specimens, Punta
 214 Piedra, 20°10'38.94"N, 87°26'42.28"W, coralline rock, 1.5 m, Feb 11, 2001, colls. S. Salazar-
 215 Vallejo, L. Carrera-Parra, M. Tovar-Hernández. **Mahahual**. ECOSUR-S67, 1 specimen, 25 m
 216 off coast, 18°43'27.09" N, 87°42'3.64"W, reef lagoon, rocky substrate with sediment, 0.75 m,
 217 Oct 1, 1996, colls. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S71, 1 specimen, in sponge,
 218 Jul 21, 1998. ECOSUR-S72, 2 specimens, 50 m off coast, 18°43'38.68"N, 87°41'56.81"W,
 219 coralline rock, 2 m, Mar 4, 1998, colls. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S73, 6
 220 specimens, reef lagoon near to back reef, 18°42'34.01"N, 87°42'31.22"W, coralline rock, 1.5 m,
 221 Jan 9, 2001, colls. P. Salazar-Silva, J. Bastida-Zavala, M. Tovar-Hernández, S. Salazar-Vallejo,
 222 L. Carrera-Parra. ECOSUR-S74, 1 specimen, fore reef, 18°42'43.32"N, 87°42'22.51"W, coralline
 223 rock, 15 m, Jun 6, 1998, coll. M. Ruiz-Zárate. ECOSUR-S75, 2 specimens, reef lagoon,
 224 18°42'36.23"N, 87°42'31.60"W, coralline rock, 1.5 m, Dec 1, 2000, colls. S. Salazar-Vallejo, L.
 225 Carrera-Parra. ECOSUR-S76, 2 specimens, reef crest, 18°43'06.17"N, 87°42'14.17"W, coralline
 226 rock, 1 m, Jul 21, 1998, colls. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S77, 5 specimens,
 227 back reef, 18°42'31.30"N, 87°42'30.39"W, coralline rock, 2 m, Mar 22, 2000, colls. S. Salazar-
 228 Vallejo, L. Carrera-Parra. ECOSUR-S78, 1 specimen, reef lagoon, 18°42'36.17"N,
 229 87°42'32.65"W, coralline rock, 1.5 m, Mar 21, 2000, colls. J. Bastida-Zavala, P. Salazar-Silva.
 230 ECOSUR-S79, 5 specimens, old wooden pier, 18°42'41.95"N, 87°42'35.98"W, fouling, 1 m, Feb
 231 24, 2001, colls. P. Salazar-Silva, J. Bastida-Zavala, M. Tovar-Hernández, S. Salazar-Vallejo, L.
 232 Carrera-Parra, L. Harris. ECOSUR-S80, 8 specimens, old wooden pier, 18°42'41.95"N,
 233 87°42'35.98"W, fouling, 1 m, Mar 18, 2001, colls. P. Salazar-Silva, J. Bastida-Zavala, M. Tovar-
 234 Hernández, S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S81, 14 specimens, reef lagoon,
 235 18°43'22.73"N, 87°42'03.08"W, coralline rock, 1.5 m, Mar 28, 2001, colls. L. Carrera-Parra, M.
 236 Londoño-Mesa, S. Salazar-Vallejo. ECOSUR-S82, 1 specimen, reef lagoon, 18°43'25.14"N,
 237 87°42'01.75"W, coralline rock, 1.5, Jan 10, 2001, colls. L. Carrera-Parra, M. Londoño-Mesa, S.
 238 Salazar-Vallejo. ECOSUR-S83, 12 specimens, reef lagoon, 18°43'24.93"N, 87°42'02.95"W,
 239 coralline rock, 1 m, Jan 19, 2001, colls. P. Salazar-Silva, J. Bastida-Zavala, M. Tovar-
 240 Hernández, S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S84, 2 specimens, reef lagoon,

241 18°43'24.93"N, 87°42'02.95"W, coralline rock, 1 m, Nov 30, 2000, colls. S. Salazar-Vallejo, L.
 242 Carrera-Parra. ECOSUR-S85, 13 specimens, reef lagoon, 18°43'21.01"N, 87°42'04.28"W,
 243 coralline rock, 1.5 m, Feb 24, 2001, colls. P. Salazar-Silva, J. Bastida-Zavala, M. Tovar-
 244 Hernández, S. Salazar-Vallejo, L. Carrera-Parra, L. Harris. ECOSUR-S93, 1 specimen, punta
 245 Rio Bermejo, 18°41'07.53"N, 87°43'05.83"W, coralline rock, 1 m, May 17 2002, colls. S.
 246 Salazar-Vallejo, M. García-Madrigal. ECOSUR-S94, 16 specimens, punta Rio Indio,
 247 18°48'29.63"N, 87°39'58.82"W, coralline rock, 1.7 m, Mar 17, 2001, coll. L. Carrera-Parra.
 248 **Xahuayxol**, ECOSUR-S58, 8 specimens, reef lagoon, 18°30'11"N, 87°45'29"W, coralline rock, 2
 249 m, Jun 1 1997, colls. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S59, 1 specimen, back reef,
 250 18°30'11.45"N, 87°45'21.46"W, coralline rock, 12 m, Oct 30 1997, colls. R. Saenz-Morales, S.
 251 Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S60, 4 specimens, reef lagoon, 120 m off coast,
 252 18°30'41.34"N, 87°45'24.63"W, coralline rock, 1.5 m, Oct 31 1997, colls. S. Salazar-Vallejo, L.
 253 Carrera-Parra. ECOSUR-S61, 11 specimens, reef lagoon, 18°30'39.77"N, 87°45'24.80"W,
 254 coralline rock, 1.8 m, Jun 4, 1998, colls. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S68, 3
 255 specimens, reef lagoon, 18°30'39.04"N, 87°45'25.09"W, coralline rock, 1.7 m, Sep 27, 1996,
 256 colls. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S95, 21 specimens, reef lagoon,
 257 18°30'12.46"N, 87°45'29.79"W, coralline rock, 2 m, Jun 1, 1997, colls. S. Salazar-Vallejo, L.
 258 Carrera-Parra. ECOSUR-S97, 1 specimen, 100 m off coast, 18°30'41.43"N, 87°45'25.16"W,
 259 coralline rock, 2 m, Sep 28, 1996, coll. S. Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S98, 1
 260 specimen, reef lagoon, 18°30'12.46"N, 87°45'29.79"W, coralline rock, 2 m, Jun 1, 1997, colls. S.
 261 Salazar-Vallejo, L. Carrera-Parra. ECOSUR-S99, 6 specimens, reef lagoon, 18°30'13.71"N,
 262 87°45'31.50"W, coralline rock, 1 m, Jun 2 1998, colls. S. Salazar-Vallejo, L. Carrera-Parra.
 263 ECOSUR-S100, 2 specimens, 100 m off coast, 18°30'15.08"N, 87°45'30.98"W, in sediment with
 264 *Thalassia testudinum*, 2 m, Sep 27, 1996, colls. S. Salazar-Vallejo, L. Carrera-Parra. **Xcalak**.
 265 ECOSUR-S101, 9 specimens, back reef, 18°15'50.40"N, 87°49'31.12"W, coralline rock, 1.7 m,
 266 Oct 25, 2002, colls. S. Salazar-Vallejo, L. Carrera-Parra, P. Salazar-Silva, M. Londoño-Mesa.
 267 **Nicaragua, Nicaraguan Caribbean**. UMML 000, 1 specimen, R/V Pillsbury, Cruise 7101, sta.
 268 1338, 12°52'00"N, 82°35'17.98"W, hillocks or low rounded mounds, 28 m, Jan 29, 1971, coll. G.
 269 Voss. **Colombia, Providence Island**. UMML 000, 1 specimen, R/V Pillsbury, Cruise 7101, sta.
 270 1349, 13°33'00"N, 81°28'00"W, patch reef slightly southwest of Low Island surrounded by sand
 271 bottom, 3 m, Jan 30, 1971. **Dominican Republic**. UMML 000, 2 specimens. R/V Pillsbury,
 272 Cruise 7006, sta. 1272, off Cabo Rojo, 17°52'41.98"N, 71°41'12.01"W, 20-27 m, Jul 17, 1970,
 273 coll. J. Staiger. **Turks and Caicos**. UMML 000. 1 specimen, Pillsbury Cruise 7106, sta. 1423,
 274 21°40'59.98"N, 71°22'59.98"W, Jul 19, 1971. **Saint Martin, Îlet de L'embouchure**. UF 329, 1
 275 specimen, 18°04'1.2"N, 63°00'39.6"W, reef flat lagoon with seagrass, 1 m, Apr 9, 2012, colls. G.
 276 Paulay, J. Slapcinsky, M. Bemis. UF 330, 5 specimens, 18°04'01.20"N, 63°00'43.20"W, reef flat
 277 lagoon with seagrass, 0-1 m, Apr 9, 2012, colls. G. Paulay, J. Slapcinsky, M. Bemis. UF 338. 1
 278 specimen, 18°04'01.20"N, 63°00'43.20"W, reef, 1 m, Apr 17, 2012, coll. A. Anker. UF 340, 1
 279 specimen, 18°04'01.20"N, 63°00'43.20"W, reef, 1 m, Apr 17, 2012, coll. A. Anker. UF 347, 1
 280 specimen, 18°04'01.20"N, 63°00'43.20"W, reef, 0-1 m, Apr 17, 2012, colls. G. Paulay, J.

Slapcinsky, A. Anker. UF 359, 3 specimens, steep rubbly reef slope, 2-10 m, Apr 22, 2012, colls. G. Paulay, F. Michonneau. **Mont Vernon**, UF 332, 1 specimen, Little Key, 18°06'00"N, 63°01'19.20"W, seagrass, sand, rocks, 0-2 m, Apr 11, 2012, coll. J. Slapcinsky. **Grande Caye**, UF 353, 1 specimen, 18°06'43.20" N, 63°01'08.40"W, coral rocks, 3 m, Apr 20, 2012, coll. A. Anker. **Rocher Créole**. UF 352. 1 specimen, 18°07'04.80"N, 63°03'21.60"W, reef, 9 m, Apr 18, 2012, colls. G. Paulay, J. Slapcinsky, M. Bemis. **NE side of St. Martin**, UF 336, 1 specimen, 18°07'48"N, 63°00'18"W, canyon with sponges, in algae, 13 m, Apr 11, 2012, coll. R. Renoux. **Île Tintammaré**, UF 361, 3 specimens, Chicot, windward side of the island, 18°06'7.2"N, 62°58'58.80"W, reef, in rubble, 12-15 m, Apr 23, 2012, coll. G. Paulay. **Caye Verte**, UF 364, 1 specimen, 18°05'24"N, 63°00'43.2"W, reef with sand and grass, 7 m, Apr 25, 2012, coll. M. Bemis. **Panama. Bocas del Toro**. UF 126, 1 specimen, coll. C. Meyer. UF 485. 1 specimen, Punta Puebla, 9°22'01.20"N, 82°17'27.60"W, May 16, 2016, colls. M. Leray, F. Michonneau, R. Lasley. UF 486, 1 specimen, Punta Juan, 9°18'03.60"N, 82°17'38.40"W, May 16, 2016, coll. R. Lasley. UF 494, 1 specimen, Runway, 9°20'31.20"N, 82°15'36"W, May 23, 2016, colls. M. Leray, F. Michonneau, R. Lasley. UF 499. 1 specimen, Marina, 9°19'51.60"N, 82°14'38.40"W, May 28, 2016, colls. M. Leray, F. Michonneau, R. Lasley. UF 500, 2 specimens, Marina, 9°19'51.60"N, 82°14'38.40"W, May 28, 2016, colls. M. Leray, F. Michonneau, R. Lasley. UF 501, 1 specimen, Cayo Hermanas, 9°16'04.80"N, 82°21'07.20"W, May 30, 2016, colls. M. Leray, F. Michonneau, R. Lasley. UF 503, 1 specimen. Cayo Hermanas, 9°16'04.80"N, 82°21'07.20"W, May 31, 2016, colls. M. Leray, F. Michonneau, R. Lasley. UF 541, 1 specimen, Salt Creek, 9°16'48"N, 82°06'07.20"W, outer reef, *Agaricia* reef, 4-4.5 m, May 22, 2016, colls. M. Leray, F. Michonneau, R. Lasley. UF 542, 1 specimen, Runway, 9°20'31.20"N, 82°15'36"W, May 23, 2016, colls. M. Leray, F. Michonneau, R. Lasley, 4-4.5 m, lagoon fringing reef, *Agaricia* reef.

Redescription

Male specimen from St. Martin, West Indies (UF 332).

External anatomy. Trunk 14 mm in length (Fig. 1A); light brown with some darker patches; papillae dark brown and light brown distributed randomly, most of them on the dorsal region, scarce ventrally; papillae conglomerated in anal region (Fig. 1D) and caudal region (Fig. 1F), most dispersed in median region (Fig. 1E).

Introvert 10 mm length (Fig. 1A), dark brown, uniform color ventrally and dorsally, with ill-defined transversal bands light brown color (Fig. 1B), distal introvert with similar color pattern as trunk, extensible collar small. Introvert papillae dark brown, smaller than those of trunk (Fig. 1C), located between rings of hooks, starting from the first ring (Fig. 2H), these are likely secretory or sensory papillae. Nuchal organ with wavy contour.

Hooks laterally compressed, arranged in sixty complete rings and some incomplete rings, probably due to abrasion. Rings followed by a zone with scattered hooks. Proximal hooks (Fig. 2A, F) with an angle of 90° between line X and Y; length of distal tip never projecting beyond

the base of the hook; secondary tooth rounded; internal clear streak (apical canal) expanded near to midpoint of vertical and middle horizontal portions of hook. Hooks of median region with a larger secondary tooth (Fig. 2B, G), distal hooks (Fig. 2C) with principal tooth smaller than its base, almost 25% less. Proximal hooks with external border bent squarely; hooks of the median region of the introvert with progressively rounder bent border; distal hooks with evenly rounded external border.

SEM revealed growth stages of hooks and papillae (Fig. 2H); smaller in the proximal introvert and proximal trunk and larger at the distal introvert and distal trunk. Introvert papillae with three stages of development. First stage: the smallest, spherical with a ring of short apical protrusions (Fig. 2K) “dome shape” (*vide* Cutler 1994). Second stage: medium size, appearance of two units, the smallest with a ring of short apical protrusions, and a broad base (Fig. 2J) “mammillate form” (*vide* Cutler 1994). Third stage: largest, conical (Fig. 2I) “cone shape” (*vide* Cutler 1994).

Internal anatomy (Fig. 2D). A pair of nephridia occupying 80% of trunk length, open at the same level as anus. Longitudinal musculature divided into 23 individual and anastomosed bands in the median trunk. Two pairs of retractor muscles; ventral pair attached to 8 longitudinal bands starting from the third band after ventral nerve cord (Fig. 2E), dorsal pair attached to 5 longitudinal bands starting from the fifth band after ventral nerve cord. Contractile vessel without swelling or villi. Spindle muscle attached posteriorly.

Habitat. In coralline rock and hard bottom, 1 to 30 m depth.

Distribution. Greater Caribbean from Florida to Venezuela.

Remarks. Keferstein (1865) described *Phascolosoma* (*Phascolosoma*) *varians* from St. Thomas, West Indies and *P. (P.) nigrescens* from Fiji. Although Keferstein’s descriptions were well illustrated, Selenka (1883) produced an improved set of drawings from the type materials (Fig. 3). Keferstein (1865) recognized each species as follows: *Phascolosoma varians* with a body three to four times as long as thick; introvert as long or longer than the body; closely spaced rows of hooks, highly variable in number (12-90), which often only cover the anteriormost part of the trunk; hooks very broad, with an upper right-angled tip (0.072 mm high, 0.092 mm wide); with 20-28 short tentacles, standing in two rows at the side; longitudinal muscles about thirty, but in many cases anastomosed as longitudinal strands; contractile vessel simple, without lateral sags; nephridia very long, attached in the anterior third by a mesentery. *Phascolosoma nigrescens* has a trunk about four times as long as thick; introvert longer than trunk; numerous hooks forming rings situated very close to each other in the anterior end of the trunk; hooks flattened with an lower right-angled tip (0.084 mm high, 0.084 mm wide); over twenty tentacles in several rows; muscles separated in about 24 longitudinal strands with few anastomosed bands; contractile vessel on the esophagus with many small, lateral sags; nephridia attached along almost their entire length by a wide mesentery.

Cutler & Cutler (1990) reviewed the subgenus *Phascolosoma* (*Phascolosoma*), and designated *P. (P.) varians*, together with 9 other species from different regions of the world, as junior synonyms of *P. (P.) nigrescens*. At that time, Cutler & Cutler (1990) suggested there were “no consistent differences” between these two species. Their decision appears to be primarily based upon variation associated with hook morphology. According to Cutler & Cutler (1990): “One possible hypothesis is that hook morphology is determined by more than one pair of genes and that allelic frequencies vary from place to place. The alleles for sharp angle and large secondary tooth occur at a high frequency in the Caribbean and a low frequency in the Indo-West Pacific. . . *P. varians* is the junior name because it was described later on the page”. No molecular evidence was ever provided in support of the allelic hypothesis, or any other genetic differences supporting proposed junior synonyms. Subsequently, Cutler (1994) published his synonymy upon further consideration of the morphological differences between *P. (P.) nigrescens* and *P. (P.) varians*.

Herein, reexamination and redescription of *P. (P.) varians*, revealed clear morphological differences concerning its previously designated senior synonym. The most important features that distinguish these two species include the shape of the hooks, the attachment of nephridia to the body wall, and morphology the contractile vessel. *Phascolosoma (P.) varians* has hooks with a rounded secondary tooth; the base of the hook is broader than high; most of the anterior hooks (Fig. 2A and 2F) possess a distal tip at a 90° angle with respect to the perpendicular axial line of the hook; the contractile vessel is simple; nephridia are attached to body wall in the anterior third. *P. (P.) nigrescens* has hooks with a square secondary tooth; the base of hook is as broad as high; most of the anterior hooks possess a distal tip with less than a 90° angle with respect to the perpendicular axial line of the hook; a contractile vessel on the esophagus with many small, lateral sags; nephridia are attached almost along their entire length.

The wavy contour of the nuchal organ and specific attachments of the retractor muscles were not described for both species by Keferstein in 1865, nevertheless, these features are now described. Keferstein (1865) refers to “tentacles in two rows or many rows”, which may explain the wavy contour of the nuchal organs I observed. The differences between the number of tentacles is not useful for separating these species because they appear to vary with the development stage of the specimen, and that variation overlaps between these species. Because of the difficulty of establishing the exact number of longitudinal muscle bands, this characteristic should be considered cautiously. Additionally, the number of rings of hooks is variable between these species, and the loss of rings is not uncommon. Papillae are also inappropriate characters for distinguishing these species as their differences are minor across most of the body surface.

Phascolosoma (Phascolosoma) granulatum was included for comparison in the molecular analyses. The species can be easily distinguished from *P. (P.) varians* for having hooks with a basal triangle and lacking bands of pigmentation in the introvert, while *P. (P.) varians* lacks a basal triangle in the hooks and the bands of pigmentation are conspicuous.

Molecular analyses

Herein, the synonymy of *Phascolosoma (P.) varians* with *Phascolosoma (P.) nigrescens* is rejected due to morphological differences. Additionally, there is a high genetic divergence between nucleotide sequences within their respective cytochrome c oxidase subunit 1 (COI) genes, which supports the morphological data.

The first three sequences (Fig. 4) (top-down) correspond to *Phascolosoma (P.) varians* from the Greater Caribbean. This species had been determined as *Phascolosoma (P.) nigrescens* by past authors and registered as such it in GenBank. These sequences were grouped with a low intraspecific variation of 2.6%. The localities of those sequences are Florida and Barbados; preserved specimens from the same localities were revised morphologically to support the correct identification of the species.

The two sequences of *Phascolosoma (P.) nigrescens* from New Caledonia constitute a group clearly separated from *Phascolosoma (P.) varians* from the Greater Caribbean (genetic mean distance 24%). There are two crucial facts to consider. First: New Caledonia is the closest locality to Fiji, the type locality of *P. (P.) nigrescens*. The high genetic divergences between the specimens from New Caledonia and those from the Greater Caribbean supports the morphological differences between both species and reinforce the reinstatement of *P. (P.) varians*. Second: the intraspecific variation between both sequences is very high (18%), almost the same value of the interspecific variation of the other groups in this analysis. This value suggests that it is highly likely that these two sequences represent different morphotypes, and either one of them would correspond with *Phascolosoma (P.) nigrescens*.

The values of the genetic distance between *Phascolosoma (P.) nigrescens* from Israel, South Africa and Broome, Australia, varies from 18 to 26% regarding *Phascolosoma (Phascolosoma) varians* from the Greater Caribbean. These values are similar to the results of Silva-Morales *et al.* (2019), where they found a genetic distance of 19% between *Antillesoma antillarum* (Greater Caribbean) and *A. mexicanum* (Southern Mexican Pacific). The present analysis reveals that *Phascolosoma (Phascolosoma) nigrescens* is a species complex resulting from the incipient morphological analysis. This study shows that *Phascolosoma (P.) varians* from the Greater Caribbean is well differentiating morphologically and genetically of *Phascolosoma (P.) nigrescens*; however, a detailed morphological revision of this species complex is needed.

Discussion

The genetic analysis suggests the following considerations: 1) the specimens from Israel, South Africa and Broome, Australia identified as *Phascolosoma (P.) nigrescens* correspond to different species. It is very likely that other species from those regions, considered synonyms, would need to be reinstated following redescription, or described and established as new species. 2) It will be necessary to resolve a potential species complex of *Phascolosoma (P.) nigrescens* by combining molecular and morphological data. Neither one of these considerations were resolved in the present study, as they were beyond the original scope of this investigation.

The assumed wide distribution of Sipuncula taxa is attributed to the development of the teleplanic larvae in some species, such as *Phascolosoma (Phascolosoma) varians*. Although it is well accepted that the free-swimming larval stage with its prominent locomotive organ (known as a metatroch) confers the ability to disperse, allowing an increase in geographic range and providing for genetic exchange between populations (Rice, 1981), it is not necessarily the rule. Staton & Rice (1999) described the case of a species with teleplanic larvae with a limited distribution. They found distinct genetic differences within and between larval and adult stages of *Apionsoma (A.) misakianum* (Ikeda, 1904) from northern and southern regions of Florida and the Bahamas using allozymes. They did not find any indication of “hybrids” occurring between them, suggesting a potential oceanic boundary was present between populations with teleplanic pelagosphera larvae, and thus a possible *Apionsoma* species-complex in the region. They did not perform a morphological analysis of the adults at that time, but the larve exhibited two distinct coloration patterns.

Kawauchi & Giribet (2010) rejected the cosmopolitanism of *Phascolosoma (P.) perlucens* Baird, 1868, by analyzing molecular and morphological data of specimens from many localities around the world. They detected four different lineages, and identified variation in hook morphologies between different localities that correlated with a high genetic diversity between populations. Also, their results suggested a probable lack of gene flow between the geographically distinct lineages. Schulze *et al.* (2012) analyzed molecular sequence data and developmental features of three “cosmopolitan” species, *Phascolosoma (P.) agassizii* Keferstein, 1866, *Thysanocardia nigra* (Ikeda, 1904), and *Themiste (T.) pyroides* (Chamberlin, 1920). For each one of the three species, they found significant differences between previously assumed con-specific populations from the Sea of Japan and the Northeast Pacific region, with respect to egg size, developmental mode and developmental timing. The populations of all three species were remarkably distinct genetically and suggested that gene flow between the two regions was extremely unlikely. Furthermore, Kawauchi & Giribet (2014) analyzed the genetic data from four genes of *Sipunculus (S.) nudus* Linnaeus, 1766 with specimens from multiple localities worldwide. As with *Phascolosoma perlucens* (see above), these two investigators again found high levels of genetic differentiation between distantly related populations, suggesting in this case there were five distinct lineages, three of which could be distinguished morphologically. In the last two studies, neither a new species was described, nor an available name reinstated.

Conclusions

High genetic divergence between specimens identified as *P. P. nigrescens* from the Greater Caribbean and a region close to its type locality correlate with morphological differences found between *P. (P.) varians* and *P. (P.) nigrescens*. Herein, the synonymy of *Phascolosoma (P.) varians* with *Phascolosoma (P.) nigrescens* is rejected, and as a consequence, *Phascolosoma (P.) varians* is reinstated.

Based upon these findings, and other studies, some of which are discussed here, the diversity of sipunculans is most likely underestimated. Thus, a combination of morphological

and molecular data, along with other important information from the fields of development, ecology and oceanography, will help us determine a more realistic number of extant sipunculans species worldwide.

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Figure 1

Papillar morphology on *Phascolosoma* (*Phascolosoma*) *varians* Keferstein, 1865

Regional variation in the patterns of papillae along the body are shown for specimen UF 332, from Saint Martin, West Indies. (A) Adult body plan in dorsal view with anterior to the top. (B) Papillae from introvert region with light transversal bands. (C) Papillae from introvert. (D) Papillae from anal region of trunk. (E) Papillae from median region of trunk. (F) Papillae from caudal region of trunk. Scale bars = A, 2 mm; B-C, 0.3 mm; D, 1 mm; E-F, 0.5 mm. White arrow indicates the location of the anus.

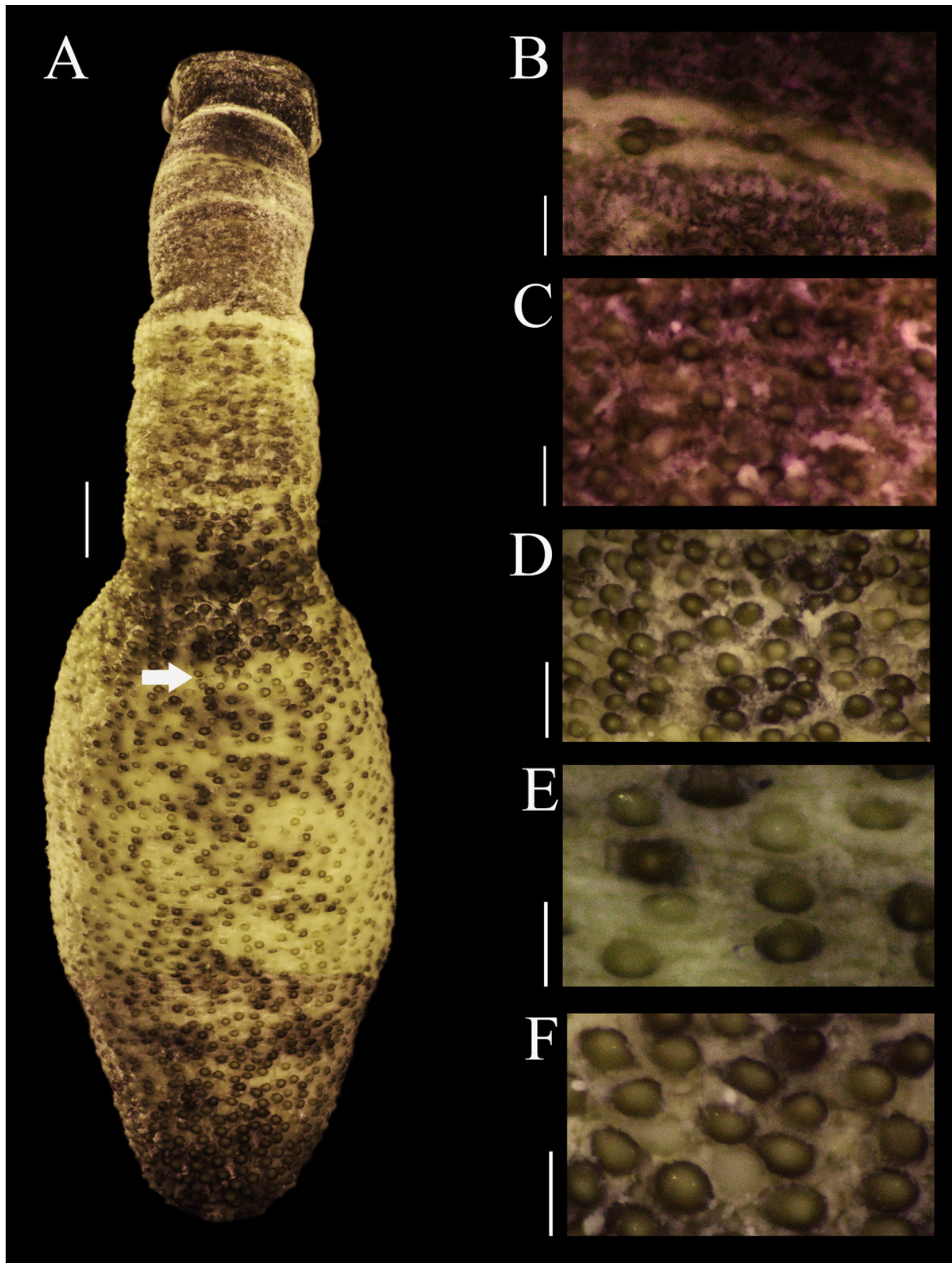


Figure 2

Anatomical characters of *Phascolosoma* (*Phascolosoma*) *varians* Keferstein, 1865

Micrographs of hook, muscle and papillar anatomy are shown for specimen UF 332, from Saint Martin, West Indies, unless stated. (A) Proximal hooks, (B) hooks of median region of introvert and (C) distal hooks. (D) Internal anatomy of a dissected specimen, with anterior to the top. (E) Posterior attachment site of a ventral retractor muscles (VRM) associated with longitudinal muscle bands 1-9; specimen ECOSUR-S94 from the Mexican Caribbean. (F) SEM image of hook from anterior region of the introvert; specimen UF359 from St. Martin, West Indies. (G) SEM image of hooks from median region of the introvert. (H) SEM image showing stages of hook growth; from left right: first seven rings of introvert hooks, arrows show individual papillae between rows of hooks. (I) SEM image of papilla from distal introvert. (J) SEM image of papilla from median region of the introvert; specimen UF 26.5 from Florida. (K) SEM image of papilla from proximal introvert. Abbreviations: DRM, dorsal retractor muscles; IN, intestine; LMB, longitudinal muscle bands; N, nephridia; SM, spindle muscle; SEM, scanning electron microscope; VRM, ventral retractor muscles. Scale bars = A, B, C, 30 μm ; F, G, I, J, 20 μm ; H, 50 μm ; K, 5 μm .

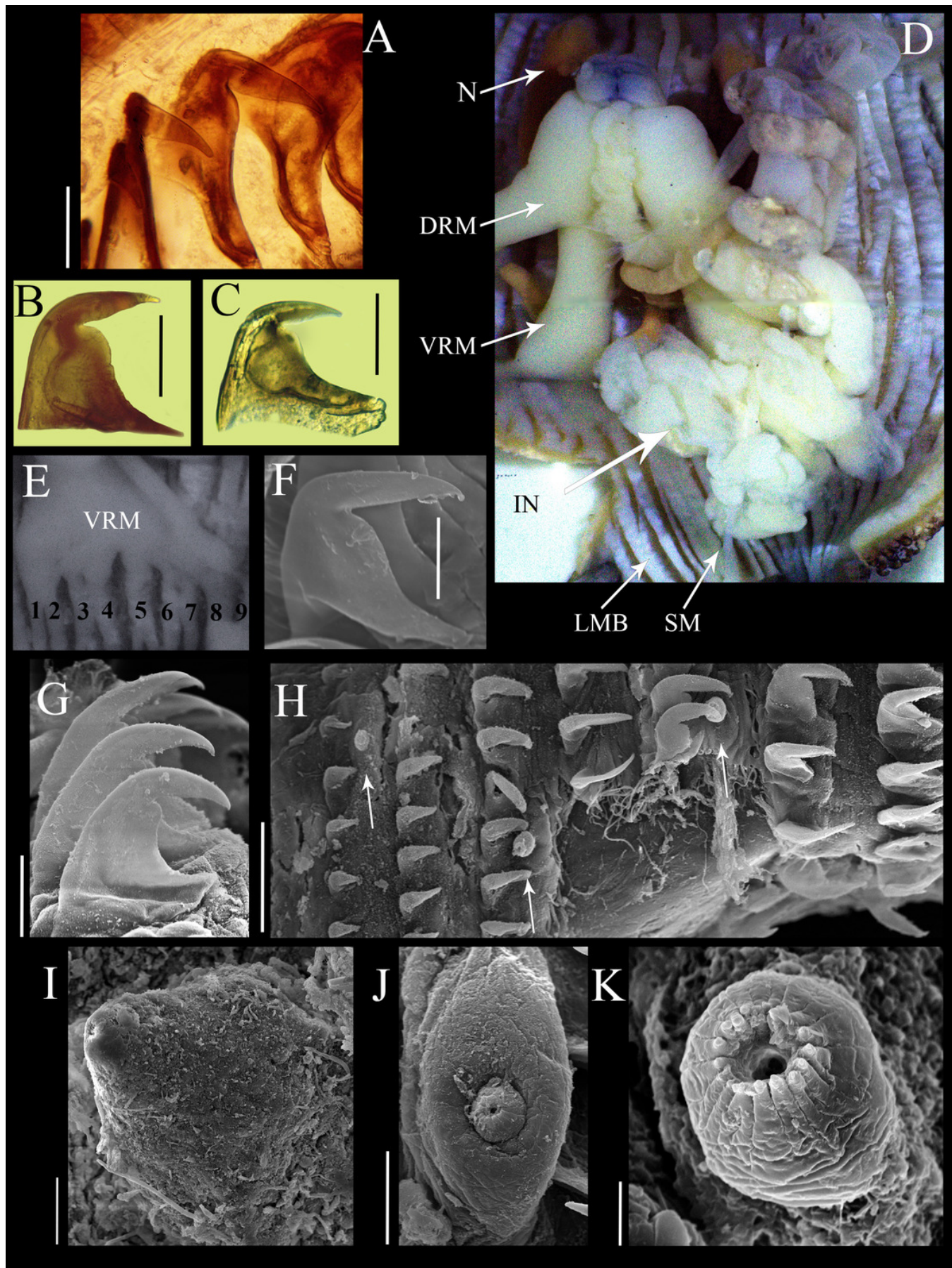


Figure 3

Hooks and papillae of *Phascolosoma varians* and *Phascolosoma nigrescens*.

From drawings of (A-D) *Phymosoma varians*, Barbados and (E-H) *Phymosoma nigrescens*, Fiji Islands, by Selenka (1883). (A) Trunk hook. (B) Papilla between the hook rings on introvert. (C) Papilla from side in longitudinal section. (D) Skin of the middle of the body with domed papillae. (E) Trunk hook. (F) Middle of papilla in 'G' from above. (G) Papilla between the scattered introvert hooks. (H) Domed body papilla at 140X magnification. Both species originally described by Keferstein, 1865.

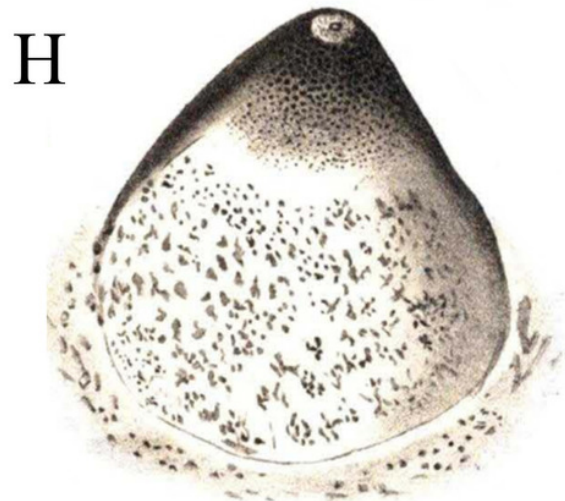
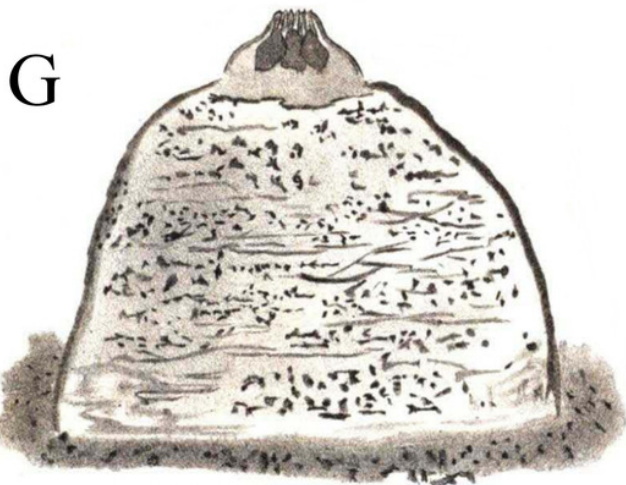
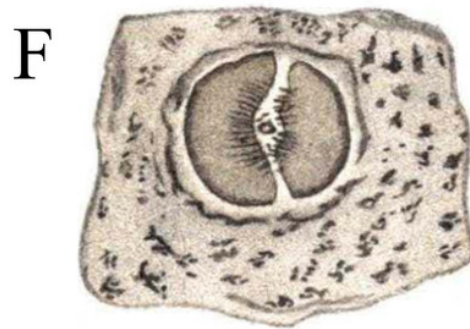
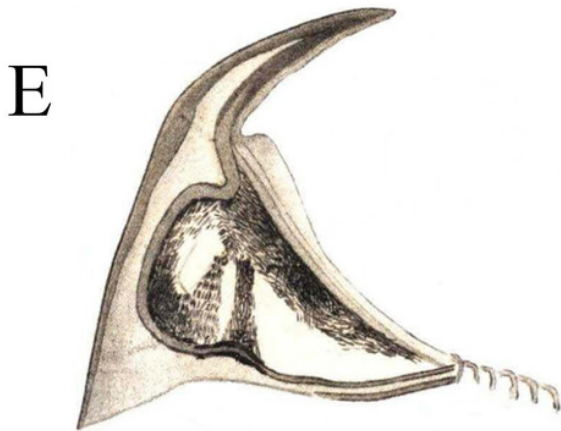
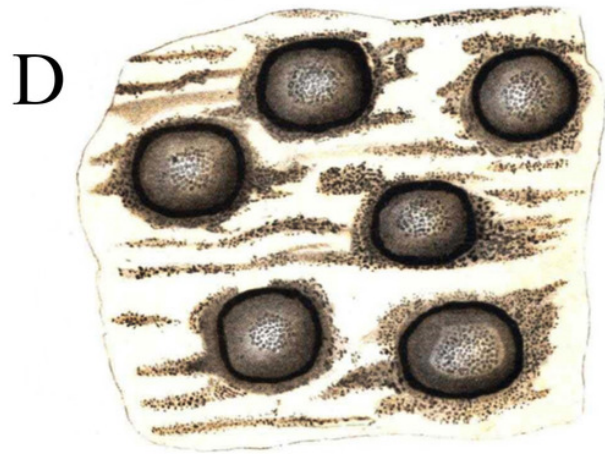
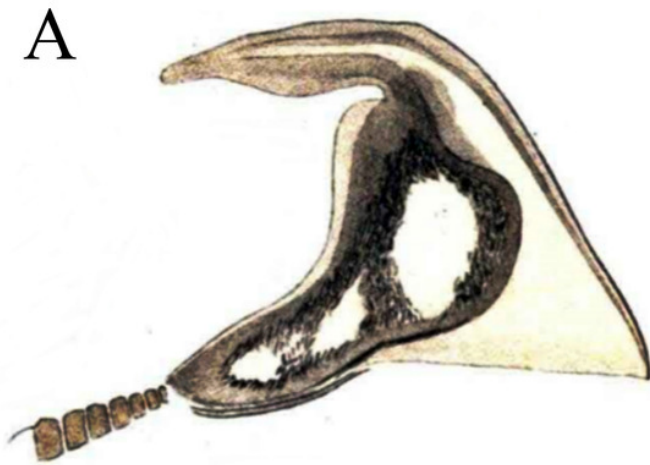


Figure 4

Maximum likelihood analysis of cytochrome c oxidase subunit 1 (COI) DNA.

Phascolosoma (P.) varians from the Greater Caribbean shows a clear genetic distinction from all specimens identified as *Phascolosoma (P.) nigrescens* from eastern and western regions of the Pacific Ocean. Tree reconstruction was generated from a ClustalW alignment of COI sequences amplified from 11 sipunculan specimens. Species names (specimens) and GenBank accession numbers are listed at the branch tips. Individuals and conspecific groups are marked with vertical bars, and their corresponding geographic regions, to the right of species names. This analysis was performed using Tamura 3-parameter with a discrete Gamma distribution with five rate categories, assuming a certain fraction of sites is evolutionarily invariable (T92+G+I).

