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Molecular identification of the Edible Chiton, *Chiton articulatus* (Polyplacophora: Chitonidae) from Mexican Tropical Pacific

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ABSTRACT
Chitons are one of the understudied classes of molluscs in Mexico. Yet, they are common and abundant inhabitants of the rocky shores that make up large parts of the coastlines along the Mexican Pacific. *Chiton articulatus* Sowerby I, 1832 is one of the most common species in Mexico and is considered as endemic to the biogeographic ecoregion of the Mexican Tropical Pacific. Specimens of *C. articulatus* were collected in the mid-upper intertidal at several localities. All collected animals were morphologically characterised, and four specimens were selected for molecular characterisation based on COI-5P and 16S sequences. The molecular data revealed that all specimens belong to the same species, *C. articulatus*. In phylogenetic trees based on both markers, these specimens represented an independent clade with *C. tuberculatus* as sister species. Our study presents the first molecular data for *C. articulatus*, a species of economic importance.

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Introduction

Molluscs have an astounding diversity in shell shapes, but chitons have a conserved shell morphology (Salloum *et al*. 2023), which has remained (in terms of its morphology) very similar to its Palaeozoic ancestral form throughout geological time (Sigwart 2009; Sigwart *et al*. 2013). Chitons comprise organisms with ecological, economic, and nutritional value (Melo *et al*. 2011; Flores-Garza *et al*. 2012; Quiroz-González *et al*. 2020) that occur from the intertidal zone to depths of 7000 m in the abyssal areas around the world (Slieker 2000; Schwabe and Wanninger 2006; Sigwart and Sirenko 2012). The identification and differentiation species in the *Chiton* genus is still largely based on morphological characteristics of the tegmentum, articulamentum, girdle and radula (García-Ríos 2003). Since the study by Okusu *et al*. (2003), efforts have been made to complement morphological knowledge with molecular data. This approach could help phylogenetic relationships in the group, validate the taxonomic status of the species and contribute data to the knowledge of the biodiversity of these organisms (Okusu *et al*. 2003; Kelly *et al*. 2008; Irrisarri *et al*. 2020; Ibáñez *et al*. 2021; Liu *et al*. 2023).

Chiton Linnaeus, 1758 has a global distribution comprising twenty-one known species (Kaas *et al*. 2006; Horton *et al*. 2023) with a distribution that spans temperate to tropical habitats. Currently, limited molecular information is available for this genus, with data for 10 of its 21 species. Based on this data we reconstructed the phylogenetic position of *C. articulatus* within the genus and family and validate its presence at the investigated localities. In addition, data have demonstrated, in some cases, the existence of important molecular variation between populations of the same species (García-Ríos *et al*. 2015; Ibáñez *et al*. 2019, 2020; Reyes-Gómez *et al*. 2022, 2023).

Of the studies that have characterised species of *Chiton* with morphological and molecular data, two of them have been done in the coast of Mexico: *C. albolineatus* Broderip & Sowerby I, 1829, and *C. virgulatus* Sowerby II, 1840 (Reyes-Gómez *et al*. 2022, 2023). A species for which there are no molecular data is *Chiton articulatus* Sowerby I, 1832, even though it is an essential element of the intertidal communities of the Mexican Pacific, where it is endemic (Ávila-Poveda *et al*. 2020). This species has a wide natural distribution and has been recorded from Socorro Island in the Revillagigedo Archipelago to Huatulco Bays, Oaxaca (Reyes-Gómez 2016). In addition to its distribution, there is evidence of its role as an herbivore and basibiont (García-Ibáñez *et al*. 2014; Álvarez-Cerrillo *et al*. 2021; Aguilar-Estrada *et al*. 2022). It represents an important fishing resource in some areas

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of the Mexican Pacific, where it is regularly consumed (García-Ibáñez *et al.* 2013). It is known as the 'sea cockroach' or 'dog's tongue'. Its exploitation has alarmed some researchers who indicated that there may be a decline in its population size because it is regularly consumed (Flores-Campaña *et al*. 2007; García-Ibáñez *et al*. 2013). Recently, Cakmak *et al*. (2022) extracted a hydrogel with a high protein content from this species that, due to its characteristics, has high potential for future use in the materials industry.

Despite the ecological and commercial importance and potential for use in industry, there is no information at the molecular level of *C. articulatus* that can help us understand the phylogenetic position within the family Chitonidae. This work presents the first sequences of COI-5P and 16S for morphologically identified specimens of *C. articulatus* in the Mexican Tropical Pacific (MTP) and contributes to the knowledge of this representative species that is exploited on the coasts of Mexico.

Material and methods

Material collection and morphological treatment

A total of 36 specimens identified as *Chiton articulatus* were sampled in the MTP. Several field expeditions were carried out to collect polyplacophoran molluscs from four locations in this region between 2014 and 2023 in different locations of the MTP (Figure 1).

The locations sampled included Socorro Island in the Revillagigedo Archipelago, Colima (18°47′9.10″N, -111°3′13.56″W), Mismaloya, Jalisco (20°31′55.87″N, 105°17′37.52″W), Playa El Palmar, Guerrero (17° 39′0.4″ N, 101°36′ 2.79″ W), and Puerto Ángel, Oaxaca, Mexico (15°39′57.63″N, 96°29′38.32″W).

Specimens were collected in the rocky intertidal zone and preserved with 70% ethanol solution in seawater; the morphological study was made with all specimens (36), part of the foot of four specimens was used for molecular analysis. Images of their general morphological features (i.e., dorsal and ventral view, disarticulated valves, girdle) were obtained with a CANON EOS REBEL T3 camera (Canon Inc. Tokyo, Japan). The specimens were taken to the laboratory and observed under a Nikon C-LEDS stereomicroscope (Nikon, Inc Konan, Minato-ku, Tokyo, Japan). The observations of the tegument sculpture and girdle derivatives were made with an Olympus CX23 optical microscope (Olympus America Inc. Center Valley, Pennsylvania, USA). The specimens collected in the different localities were vouchered into the Colección de Invertebrados Asociados a Macroalgas from Facultad de Ciencias, Universidad Nacional Autónoma de México (FC-UNAM).

The four specimens used in the molecular analyses represent all locations sampled (one per location), because they had the morphological characteristics of *C. articulatus*, the appropriate size to obtain tissue to carry out the molecular study. Total genomic DNA was extracted using the QIAGEN DNeasy Blood & Tissue kit (Qiagen, USA) of a small portion of the foot (1 cm^2) of the four specimens. PCR reactions were set up with the Thermo Scientific Phire Plant Kit using the following reagent volumes: 10 µl of 2X buffer, 1.0 µl of each forward and reverse primer at 10 pmol, 0.4

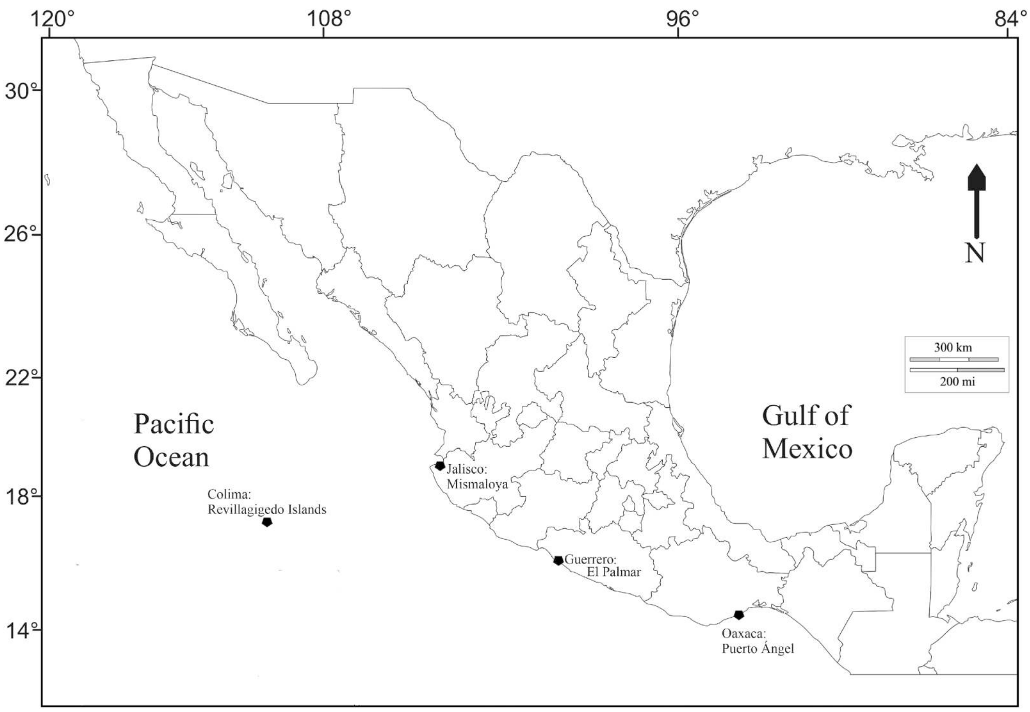

Figure 1. Map of Mexico showing the localities studied at Mexican Tropical Pacific coasts. Colima: Socorro Island in the Revillagigedo Archipelago, Jalisco: Mismaloya, Guerrero: Playa El Palmar. Oaxaca: Puerto Ángel.

µl of Taq Polymerase, 1.0 µl of DNA, and 11.6 µl of injectable water, for a final volume of 25µl. The primers used for amplifying and sequencing COI (forward: 5′-TCWACAAATCAYAAAGATATTGG-3′ and reverse: 5′-ACYTCMGGRTGMCCAAAAAATCA-3′) and 16S (forward: 5′-CGCCTGTTTATCAAAAACAT-3′ and reverse: 5′CTCCGGTTTGAACTCAGATC-3′) were those presented in Owada *et al.* (2013) and Xiong and Kocher (1991) . The reactions were run in a Techne Flexi-gene thermal cycler (Bibby Scientific, Maryland, USA) following the cycling protocol described by Owada (2016). The PCR products were sequenced by Universidad de Chapingo (Mexico).

Sequences were edited with the Bioedit (Hall 1999) or Sequencher (Gene Codes Corporation, Ann Harbour, MI, USA) programs and aligned with Clustal W (Thompson *et al*. 1994). The COI alignments consisted of four new (this study) and ten GenBank sequences for the other species of chitons (Table 1). The 16S alignments consisted of four new (this study) and seven GenBank sequences for the other species of chitons (Table 1). For both markers, *Tonicia forbesii* Carpenter, 1857, and *T. chilensis* Frembly, 1827, were designated as outgroups.

The software MEGA 7.0 (Kumar *et al*. 2016) was used to calculate genetic distances (Tables S1-S2). The evolutionary model for phylogenetic analyses was GTR + G + I for both data sets, selected as the best fit model with the Bayesian Information Criterion in JModelTest v2.1.10 program (Dariba *et al*. 2012). Phylogenies of COI datasets were inferred using maximum likelihood (ML) and Bayesian inference (BI). The ML analyses were reconstructed using RAxML v8.0.X (Stamatakis 2014) set as follows: a rapid bootstrap analysis and search for the best scoring ML tree in one single program run with 1,000 bootstrap replicates under the GTR + G + I substitution model. BI was performed for individual datasets with MrBayes v3.2.1 (Ronquist *et al*. 2012) using the Metropolis coupled Markov chain Monte Carlo under the GTR + G + I model. For each matrix, two million generations of two independent runs were performed with four chains and sampling trees every 100 generations. The burn-in period was identified graphically by tracking the likelihood at each generation to determine whether they reached a plateau. Twenty-five per cent of the saved trees were removed, and the remaining trees were used to calculate Bayesian posterior probabilities.

Results

Molecular phylogeny based on COI

The COI alignment consisted of 16 sequences (four obtained in the present work) with 560 bp. ML (Figure 2) and BI analyses produced trees with similar topologies for this marker. The *Chiton articulatus* specimens formed a clade supported by high posterior probability and bootstrap values (1 and 100, respectively). The clade was separate from the clades formed by the other species of *Chiton* from Mexico and other sites of the world. In the tree obtained with COI, the sister species of *C. articulatus* was *C. tuberculatus*, and the branch presented high values of support. For COI-5P, genetic distances among the specimens were 0.0% at the intraspecific level

Table 1. Species, collection sites, GenBank accession numbers and references with which the present work was carried out.

Species	Location	Accession GenBank		Reference
		COI	16S	
Chiton albolineatus Broderip & Sowerby I, 1829	Sayulita, Mexico	MZ073998	–	Reyes-Gómez *et al.* (2022)
Chiton articulatus Sowerby I, 1832	Playa El Palmar, Mexico (17°39′0.4″N, 101°36′ 2.79″W)	**OQ542791**	**OQ836589**	This study
	Isla Socorro, Mexico (18°47′9.10″N, -111°3′13.56″W)	**OQ542792**	**OQ836591**	This study
	Mismaloya, Mexico (20°31′55.87″N, 105°17′37.52″W)	**OQ542793**	**OQ836592**	This study
	Puerto Angel, Mexico (15°39′57.63″N, 96°29′38.32″W)	**OQ542794**	**OQ836590**	This study
Chiton barnesii Gray, 1828	El Sauce, Chile	MK016435	-	Ibáñez *et al.* (2020)
Chiton cumingsii Frembly, 1827	Antofogasta, Chile	MK016399	MK097378	Ibáñez *et al.* (2019)
Chiton glaucus Gray, 1828	Cape Pallister, New Zealand	MK016469	MK097381	Ibáñez *et al.* (2020)
Chiton granoradiatus Leloup, 1937	Tiruchendur, India	KY235767	-	Nisha *et al.* (2016)
Chiton granosus Frembly, 1827	Rio Seco, Chile	MK016404	-	Ibáñez *et al.* (2020)
Chiton magnificus Deshayes, 1827	Valdivia, Chile/ Puerto del Hambre, Punta Arenas, Chile	MK016408	MK097376	Ibáñez *et al.* (2019)
Chiton stokesii Broderip, 1832	Santa Rosalía, Ecuador	MK016411	MK097388	Ibáñez *et al.* (2019)
Chiton tuberculatus Linnaeus, 1758	Pokiok Rd, Bermuda	KJ909661	-	García-Ríos (2015)
Chiton virgulatus Sowerby II, 1840	Bahía San Felipe, Mexico	MZ073996	-	Reyes-Gómez *et al.* (2023)
Rhyssoplax kurodai (Is. Taki & Iw. Taki, 1929)	Kanagawa, Japan	-	LC071581	Owada (2016)
Tonicia chilensis (Frembly, 1827)	Punta de Tralca, Chile	MK016441	-	Ibáñez *et al.* (2019)
Tonicia forbesii Carpenter, 1857	Mazatlan, Mexico	KJ574101	-	Irisarri *et al.* (2014)

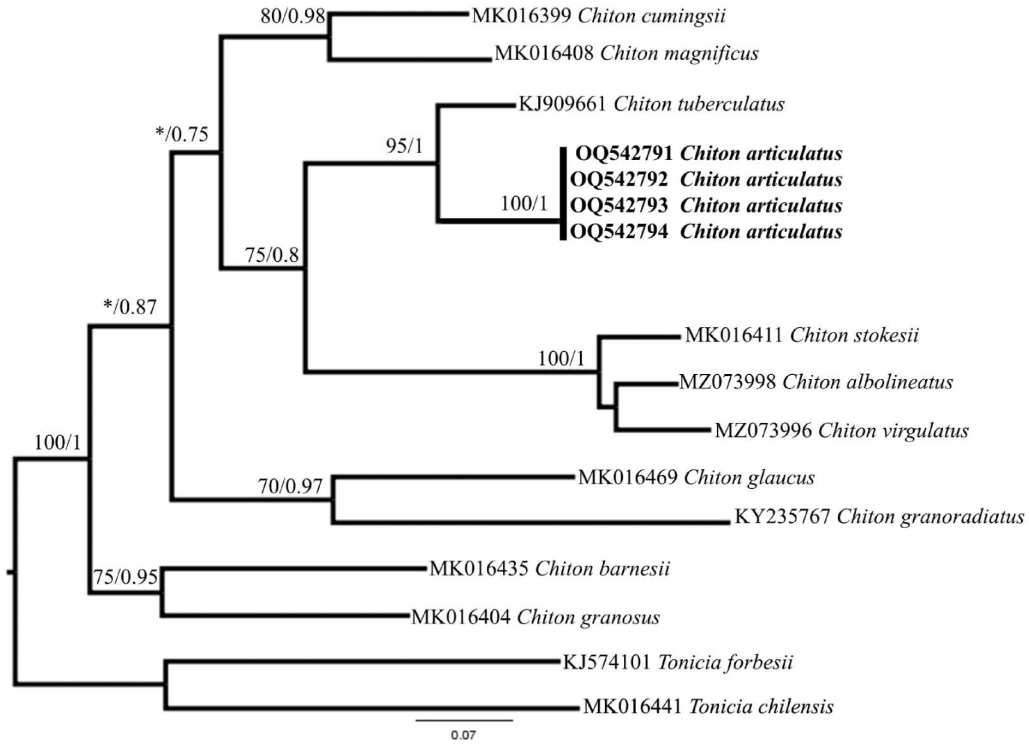

Figure 2. Maximum-likelihood tree inferred from COI-5P sequences of *Chiton*. ML bootstrap and Bayesian posterior probabilities values are shown at branches. Bold letters indicate newly generated sequences in this study.

(Figure 2, Table S2). When comparing these specimens with *C. tuberculatus*, the distances were 14.79%, and the distance to the other species of *Chiton* were 19.87–24%. (Table S2).

Molecular phylogeny based on 16S

The 16S alignment consisted of 12 sequences (four obtained in the present work) with 445 bp. ML (Figure 3) and BI analyses produced trees with similar

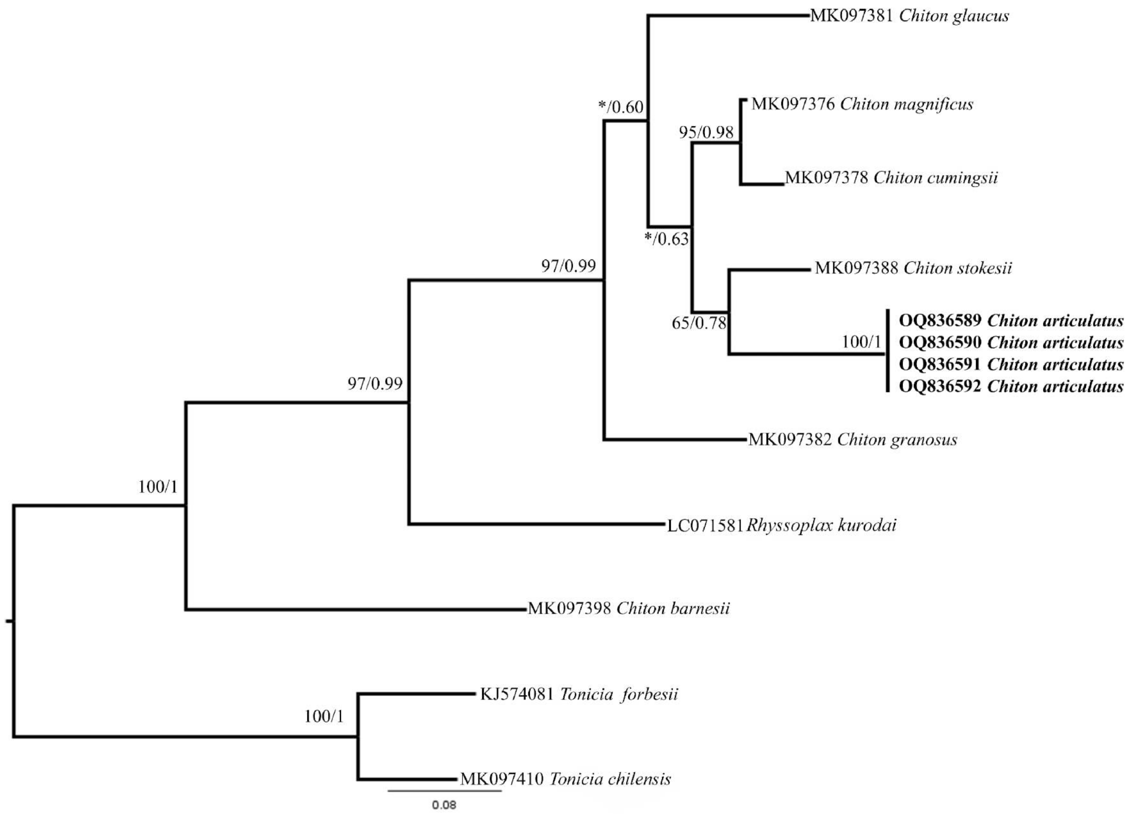

Figure 3. Maximum-likelihood tree inferred from 16S sequences of *Chiton*. ML bootstrap and Bayesian posterior probabilities values are shown at branches. Bold letters indicate newly generated sequences in this study.

topologies for this marker. The specimens of this work formed a clade supported by high posterior probability and bootstrap values (1 and 100, respectively). The clade formed by these specimens of *C. articulatus* was separate from the clades formed by the other species of *Chiton* from Mexico and other sites of the world. In the tree obtained with 16S, the sister species of *C. articulatus* was *C. stokesii*; however, the support values of the branch were very low (65/0.78) for both analyses. It should be noted that there is no *C. tuberculatus* sequence for this gene.

For 16S, genetic distances among the specimens were 0.0% at the intraspecific level. When comparing these specimens with *C. stokesii*, the distances were 10.6%, and when compared to the other species of *Chiton* distance were 10.8–21% (Supplementary file) (Table S1).

Morphology

According to the morphological revision of the characteristics of the tegument sculpture and those derived from the girdle, the specimens collected in this study were taxonomically determined as *Chiton articulatus*. This taxonomic determination was made because these characteristics are consistent with the morphological information previously collected by other authors (Table 2).

Family Chitonidae Rafinesque, 1815
Subfamily Chitoninae Rafinesque, 1815
Genus *Chiton* Linnaeus, 1758
***Chiton articulatus* Sowerby I, 1832**
(Figure 4)

Description. Oval organisms up to 100 mm length in adults. Smooth yellowish tegmentum to olive green, sides of the valves brown to green, sometimes showing dark concentric bands. Cephalic valve with a slight and strongly convex slope. The intermediate valves are smooth, the lateral margins are rounded, and the posterior margin is straight with a small and discrete apex. The apophyses are wide and short. The tail valve is wide, with a right front margin. Mucro is almost anterior. The jugal sinus is deep, the clefts are shallow, and the insertion teeth are short. The girdle is wide, with bands of green and pale yellow, and it has diamond-shaped scales.

Distribution. From Mazatlán, Sinaloa to La Ventosa, Oaxaca, including Isla Socorro, Revillagigedo, Mexico (Reyes-Gómez 2016).

Type specimens. According to Bullock (1988) and Kaas *et al.* (2006), the holotype is in the British Museum of Natural History.

Type locality. Guaymas, Sonora, Gulf of California.

Material examined. A total of 36 specimens, maximum size of 100 mm in length and 37 mm wide (INV985, INV2944-2978).

Habitat. This species is abundant along the MTP. It lives in the rocky intertidal zone. It can be observed on the rocks or between the crevices that form between them and in macroalgae assemblies.

Discussion

In the present work, the phylogenetic analysis and the genetic distances suggest that the specimens from four different localities of the MTP belong to the same taxonomic entity that coincides with the morphology described for *C. articulatus* (Broderip 1832; Keen 1971; Bullock 1988). No differences were detected between individuals from the different populations with both markers. It is necessary to evaluate of a larger number of individuals from different populations to detect intraspecific genetic variability, determine the presence of haplotypes and evaluate gene flow between populations,

Table 2. Morphological comparison between *Chiton articulatus* of this work against previous studies.

Shape	Broderip (1832) Oval	Keen (1971) Large oval	Bullock (1988) Large	This Study Oval
Size (mm)	L = 51	L = 100	L = 104	L = 100
Tegumentum	Smooth	Smooth	Smooth	Smooth
Shell color	Greenish brown, pale variegated	Grey to brown olivaceus	Off-white to yellowish or olive green	Yellowish to olive green
Valve I	-	-	Convex	Convex
Jugal and central	Almost obliterated	Tan dull orange, with broad, black longitudinal lines	Longitudinal dark brown lines	Longitudinal dark lines
Lateral areas	-	Greyish tan to slate-brown, often with fine, radiating darker lines	Yellowish brown to bluish green, occasionally with a few darker concentric bands	Brown to green, sometimes showing some dark concentric bands.
Mucro	-	-	Mucro obsolete, anteriorly acentric on posterior valve.	Mucro almost anterior.
Girdle	-	-	With alternately banded light and dark green with scales moderate in size, somewhat	Moderate in size, apparently banded pale yellow and green, with diamond-shaped scales

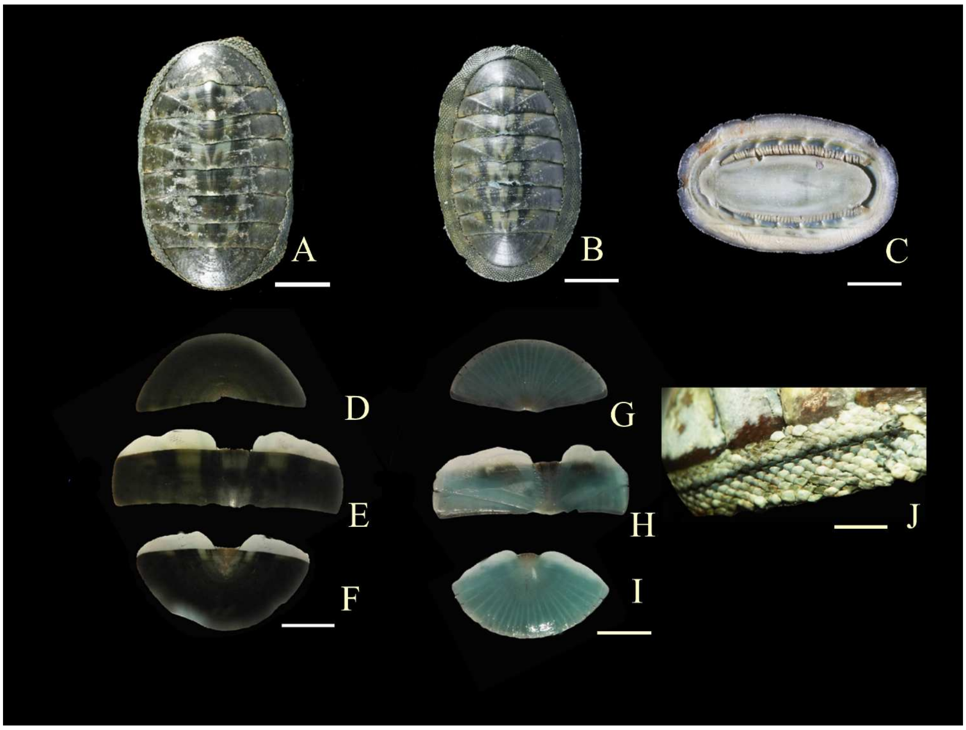

Figure 4. Morphology of *Chiton articulatus*. A, Dorsal view; scale = 10 mm. B, Dorsal view of specimen; scale = 10 mm. C, Ventral view of specimen; scale = 10 mm. D–F, Disarticulated valves in dorsal view. D, Head valve. E, Intermediate valve IV. F, Tail valve; scale = 7.5 mm. G–I, Disarticulated valves in ventral view. G, Head valve. H. Intermediate valve IV. I, tail valve; scale = 7.5 mm. J, Characteristics of girdle; scale = 3 mm.

as has been done with other species of *Chiton olivaceus* (Fernández *et al*. 2015). With the COI-5P marker and the existing data for other species of the genus (ten species), *C. articulatus* was phylogenetically closer to *C. tuberculatus*. With the 16S marker, the greatest phylogenetic closeness was exhibited with *C. stokesii*. It is worth mentioning that the number of sequences available for this marker is smaller than for COI-5P, and that the support values for this case are very low, so it is possible that some other species for which there is no molecular information are closer to *C. articulatus* than *C. stokesii*, similar to what occurs with *C. tuberculatus* for the COI-5P gene.

Pilsbry (1893) and Ferreira (1983) noted the similarity between West Indian *C. marmoratus* Gmelin, 1791, and *C. articulatus* for the Eastern Pacific. Both should be considered as sister species because they diverged from a common ancestor after the formation of the Panamanian land bridge in the early Pliocene. For this reason, New World Chitoninae have species closely related to *C. articulatus* and *C. tuberculatus* between the Atlantic and Pacific oceans (Bullock 1988). The importance of molecular biology work with species of the genus *Chiton*, especially *C. marmoratus*, is reiterated to understand the phylogenetic relationships with *C. articulatus* and other species within this genus.

It is important to point out that of the 21 valid species registered for the genus *Chiton* in the world, there is information from the COI-5P marker for ten of them and six for 16S (Irrisari *et al*. 2014; García-Ríos 2015; Nisha *et al*. 2016; Ibáñez *et al*. 2019, 2020; Reyes-Gómez *et al*. 2022, 2023), which shows that it is necessary to continue with molecular studies with this group. The values of genetic distance at the intraspecific and interspecific level for the COI-5P marker were 0.0% and 12–21% respectively. The range of interspecific distance is similar and even higher than the levels indicated by other authors for other members of the genus *Chiton* and the family Chitonidae, which range between 11% and 19% for COI and 8.6% and 18.1% for 16S (Chen and Sun 2013; Ibáñez *et al*. 2019, 2020).

A comparison of the morphological characteristics of all specimens examined in this study with the descriptions of other authors (Broderip 1832; Keen 1971; Bullock 1988), found several similarities for the tegument sculpture, colouration, and characteristics of the girdle. Differences were observed with respect to the size of the island specimens because they presented the largest sizes with respect to those that have been collected in continental areas of the MTP. These data coincide with those registered by Holguín-Quiñones and Michel-Morfin (2002) for specimens of *C. articulatus* collected on Socorro Island of the Revillagigedo Archipelago. However, the size differences of *C. articulatus* in continental specimens could also be the result of external factors, such as selective predation by tourists or fishermen, because this species is locally consumed (Flores-Campaña *et al*. 2007; García-Ibáñez *et al*. 2013; Olea-de la Cruz *et al*. 2013).

The present study contributes to the knowledge of the genetic identity of *C. articulatus* and gives an approximation of its phylogenetic relationships. However, further molecular data are needed for the remaining species in the genus. Furthermore, the importance of developing population studies on *C. articulatus* to know its genetic status is highlighted because it is a species subject to commercial exploitation.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Authors' contributions

LGAE carried out the morphological characterisation and fields work, search and prepared the figures. NQG and MEPM carried out the molecular characterisation and performed the phylogenetic trees. IRB collaborated in the field trips. All authors agreed, wrote, and revised the latest version of the manuscript.

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