

Article

Old Taxonomy Masks the Phenotypic Diversity of *Pleurobrachia* Fleming (Ctenophora: Tentaculata): An Analysis of the Morphological Variation in the Genus from Mexican Coasts

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Abstract

Pleurobrachia pileus and *P. bachei* are among the most frequently recorded ctenophores worldwide. Despite their long-standing descriptions dating back nearly 250 years, their taxonomic boundaries remain ambiguous due to the lack of definitive diagnostic features. In Mexican waters, records of both species are primarily based on geographic distribution, with limited morphological evidence supporting their separation. This study aimed to evaluate the morphological diversity of *Pleurobrachia* spp. along the Mexican coast through a comprehensive morphological approach. Specimens from four sites representing two marine regions (the Gulf of Mexico and the Southern Mexican Pacific coast) were examined for phenotypic variation. A standardized methodology was developed for specimen handling, observation, and digital reconstruction of body plans, enabling the extraction of 38 discrete and continuous morphological traits. Our analysis revealed two distinct and internally consistent phenotypic groups, each corresponding to one of the two regions, with notable differences in tentacular sheath structure, tentacular canals, gastrovascular cavity pigmentation pattern, and pharyngeal morphology. A clinal pattern in body size was observed, with smaller specimens in the north and larger ones in the south. Importantly, neither phenotype matched the diagnostic traits of *P. pileus* or *P. bachei*, suggesting that the Mexican populations represent separate, previously unrecognized species. These findings highlight the necessity to re-evaluate the taxonomy of *Pleurobrachia* in these regions and contribute valuable morphological data toward resolving longstanding questions about species boundaries within this genus.

Keywords: comb jellies; morphotype; morphological analysis; Gulf of Mexico; Southern Mexican Pacific coast



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1. Introduction

The phylum Ctenophora, commonly known as comb jellies, is a group of marine invertebrates that is well-supported by particular synapomorphies, making them a unique and evolutionarily intriguing group within the metazoan [1,2]. The combination of morphological traits displayed by this taxon, together with extensive sequence data, has provided alternative hypotheses about its phylogenetic position [3–9].

Although the phylum has been the focus of many systematic analyses, its internal classification is debated, and the group presents many problems in alpha taxonomy [2,10,11]. Fragility and the lack of hard structures for identification make their morphological traits difficult to preserve and study [12,13]. Some species disintegrate upon collection, meaning their true morphology is only visible in live observations (e.g., using remotely operated vehicles (ROVs) or deep-sea cameras). Also, standard formalin or ethanol preservation alters their delicate structures, making morphological comparisons unreliable [14,15]. Many species display wide phenotypic variation (size, color, and body structure) due to both environmental (diet or environmental stress) and ontogenetic (body shape based on age) factors, leading to misidentifications [12,16–18]. Over time, several authors have described and compared Ctenophora species based on a few specimens that are no longer available for their review in the collections where they were stored [10,17,19–24], making comparisons difficult. Some taxa were named multiple times due to poor descriptions or lack of comparative material, leading to synonymy issues within the phylum [2,13]. Current molecular studies (DNA barcoding and genomics) suggest that many Ctenophora species harbor cryptic diversity [25–27].

The species delimitation of Ctenophora requires establishing a methodology to obtain replicable and comparable diagnostic traits, defined under standardized conditions, and considering geographical variation. Moreover, the integration of additional biological and molecular data is crucial for refining species boundaries and supporting new species descriptions. These efforts will help to improve the identification of ctenophores worldwide, even by non-specialists, despite the challenges posed by specimen damage and degradation during collection and preservation [12,13,28,29].

In this context, *Pleurobrachia* (class Tentaculata, order Cydippida, family Pleurobrachiidae) is known to be the most studied comb jelly genus [6,9,30,31]. It was the second added in Ctenophora with the largest number of geographical records worldwide [12,32]. Members of this taxon were first reported as polar organisms [16,33,34] and were characterized by at least five diagnostic attributes: 1. spherical or oval body shapes; 2. eight symmetrical comb rows; 3. two retractile, branched (tentilla), and long tentacles; 4. an apical sensory organ in the aboral region, and 5. a gastrovascular cavity (GVC) elongated towards the oral region [2,17,19,23]. Under this definition, eight species have been compiled and recognized [11,13]: *Pleurobrachia pileus* Müller (1776), recorded in the northern and southern Atlantic Ocean and Arctic Ocean; *P. bachei* Agassiz (1860), from the northern Pacific Ocean; *P. rhodopsis* Chun (1879), from the Mediterranean Sea and adjacent seas; *P. pigmentata* Moser (1903), from the eastern Pacific Ocean; *P. cyanea* Chun (1889), from the eastern Atlantic Ocean; *P. globosa* Moser (1903) and *P. pigmentata* Moser (1903), from the Dutch East Indies marine region; *P. striata* Moser (1908), from the eastern tropical Pacific Ocean, and *P. brunnea* Mayer (1912), from the western North Atlantic and eastern Pacific Oceans.

In the past 25 years, many new geographical records based on other biological studies of this genus have been made outside their original distribution, supporting *P. pileus* and *P. bachei* as the most commonly recorded species around the world [12,13,30,34–46]. The distribution of *P. pileus* is estimated to be extremely wide primarily in the Atlantic and Indian Oceans, ranging from polar regions [10,47,48], cold regions [34,49–51], temperate regions [52,53], to both subtropical [46,54] and tropical regions [55,56]. Conversely, *P. bachei*

is recognized as the only species of the genus found in the Pacific Ocean in cold and temperate regions from Canada to California [31,57–59].

Although these two species were described almost 250 years ago, their boundaries are unclear, making their recognition difficult. The first description of *P. pileus* [33] only considered the spherical body shape, the symmetrical presence of the comb rows, and the position of the tentacles, as well as the geographic distribution (polar region of the Atlantic Ocean) as diagnostic attributes, while the description of *P. bachei* [19] was supported by the general body shape, which is ovoid, a size not larger than 2 cm long, the exit of the tentacular canals further away from the aboral pole, and a shorter gastrovascular cavity compared to *P. rhodopis* and *P. globosa*. The validity of these two species remains doubtful due to the lack of clear diagnostic features for their recognition. Since their original descriptions, the most heavily weighted criterion for taxonomic assignment has been geographic distribution. However, morphological attributes have proven to be ambiguous and often lead to questionable identifications, especially when based on poorly preserved specimens, photographs from social media, diver-generated pictures, or individuals of intermediate size in relation to the previously described species [20].

In Mexican coasts, geographical records of ctenophores suggest the presence of at least two species, probably allopatric. In this sense, *P. pileus* has been recorded in the coastal lagoons of La Mancha, Mandinga, and Ostión in Veracruz into the Gulf of Mexico [60], while *P. bachei* records are from coasts of Sonora, Sinaloa, Nayarit, and Jalisco in the Pacific Ocean [61–64]. The identification and distribution of *P. pileus* and *P. bachei* in Mexico seems to be related to the Atlantic and Pacific marine regions, respectively, but due to the problem of identifying them, there are many more records only at the genus level [65–71]. Although the species *P. pileus* has been recognized with the 18S gene in the northern region of the GoM, *Pleurobrachia* sp. was also identified in the same area, which could mean that more than one species occurs in this region [6]. Also, there is a record in the Mexican Pacific coast assigned to the species level, using a nomenclatural combination not recognized within the taxonomy of the phylum, which is *P. pileus* var. *bachei*, without morphological or molecular evidence to support it [72]. The most recent checklist of Mexican ctenophores adopted a geographical criterion to species assignment, which suggests that all records of *Pleurobrachia* from the Pacific Ocean and Gulf of California should be considered as *P. bachei*, while those records from the Gulf of Mexico and Caribbean should be considered as *P. pileus* [73], all of this without providing morphological evidence of the features shared for these species in the descriptions and taxonomic keys.

In this study, our objective was to evaluate the morphological diversity of *Pleurobrachia* spp. distributed along the Mexican coast based on a morphological approach. To achieve this, we developed a standardized method for specimen manipulation, observation, and digital reconstruction of different body plans to obtain comparable morphological traits among a comparable number of specimens from various locations. We examined phenotypic variation in specimens from four sites representing two marine regions (Gulf of Mexico and Southern Mexican Pacific coast). Therefore, we proposed a morphological character matrix comprising 38 discrete and continuous attributes to analyze phenotypic variation within and among populations to assess whether this variation supports the existence of at least two distinct morphological species, as previously suggested. In addition, we tested whether the morphological variation observed in Mexican specimens corresponds to either *P. pileus* or *P. bachei*.

2. Materials and Methods

A total of 120 specimens of *Pleurobrachia* spp. from four localities along the Pacific and Gulf of Mexico coasts were examined. Of the total sample, 90 were from the Gulf of Mexico

(GoM) and 30 from the Southern Mexican Pacific coast (POcM). Sample sites represented the distribution of the genus in the GoM and a reference at the Pacific coast. The Tamiahua estuary (THVZ) was a sandy beach area with a depth of no more than 1 m, fishing took place in an area of approximately 150 m² during the morning, and the surface water temperature was 33 °C, while the salinity was 31. Also, several fish larvae, jellyfish, and drifting algae were observed. In the Tampamachoco lagoon (TCVZ), fishing was carried out on a rocky and muddy beach at a depth of less than 1.3 m, many oyster shells were on the bottom, the fishing area was approximately 120 m² and near the junction with the Tuxpan River, and this sampling was carried out in the afternoon with a surface water temperature of 28 °C and salinity of 29. Some gelatinous competitors were observed, although there were fewer fish or other zooplankton members. In Balzapote bay (BZVZ), fishing was carried out in a sandy and rocky bay, near a dock, in an area of approximately 200 m², and at a depth of less than 1.5 m during the morning. The surface water temperature was 33 °C, while the salinity was 35; the water was clear, with some fish, eggs, mollusk larvae, sciphozoans, and salps. Finally, in Madero port (MTCH), fishing was carried out on a sandy tourist beach near an abandoned dock with a depth of less than 1.2 m and in an area of around 200 m², where the water was turbid with the presence of scifomedusae and hydromedusae. There were some fish and annelids; the surface water temperature was 32 °C, while the salinity was 34 (Table 1). Specimens were collected with a fish hand net (1 m long, 50 cm wide, 500 µm mesh size) near the intertidal zone as described above.

Table 1. Regions, localities, dates, sample size, and keys of collected *Pleurobrachia* specimens.

Region	Locality	Geographic Coordinates		Date	n	Key *
Gulf of Mexico	Tamiahua estuary, Veracruz	21°15'36.8" N	97°26'04.3" W	February 2023	30	THVZ
Gulf of Mexico	Tampamachoco lagoon, Veracruz	20°58'25.8" N	97°20'22.2" W	June 2022	30	TCVZ
Gulf of Mexico	Balzapote bay, Veracruz	18°37'15.4" N	95°04'09.9" W	August 2023	30	BZVZ
Pacific Ocean coast of Mexico	Madero port, Chiapas	14°42'15.2" N	92°23'55.6" W	February 2024	30	MTCH

* Key for localities: the first two letters correspond to the location; the last two letters correspond to the state.

The taxonomic identification to the genus level of field-collected specimens was carried out using available descriptions, whereby all specimens collected were spherical, with eight rows of combs arranged symmetrically around the body, a statocyst in the aboral region, an elongated gastrovascular cavity (GVC) in the oral region, and two branched tentacles [10,16,21,23]. This identification was carried out before fixation by taking photographs and making observations in the field with a camera with a microscopic lens of 2 MP, f/3.3; FOV 65°; lens 3P + IR; FF.

Individuals were fixed in formalin/2% seawater and stored at 4 °C until their manipulation in the laboratory, where we developed a method for the observation of the specimens using handmade silicone pools in a microscope slide to maintain an aqueous medium (Formalin/2% seawater–glycerin) to manipulate ctenophores for pictures. This medium reduced the flux of liquid and avoided the movement of the specimens. Using this method, reconstructions from oral/aboral and tentacular/esophageal planes were obtained from each specimen. Plane reconstructions corresponded to single sharp images obtained from at least 30 focal depths of the respective planes, automatically combined successively (i.e., focus stacking), using a digital RisingCam 16 MP attached to a RisingTech UrCMOS stereoscope and the RisingView software V2. The external and internal morphologies of specimens were exhaustively examined for both plan reconstructions, and measure-

ments were taken using the same program. Morphological terminology was adapted from previous ctenophore descriptions [10,17,19,22,23].

To analyze morphological variation in the specimens, 38 discrete and continuous morphological traits were considered. We integrated previous attributes from the available literature ($n = 10$) and evaluated 14 new ones. A total of 24 discrete attributes were considered, 6 from the external structures (comb rows, tentacles, tentilla, and pharyngeal folds) and 18 from internal structures (gastrovascular cavity, internal canals, statocyst, stomach and tentacle sheaths). Total traits were coded as double- or triple-state, and the first four were analyzed in situ, while the others were analyzed after fixation. Attributes 1–3, 5–7, 10, 12, 13, 24 have been commonly used in the literature for describing species within this genus [10,17,19,22,23], while the rest (4, 8, 9, 11, 14–19, 20–23) are newly proposed in this study (Figures 1 and 2). These characters are as follows:

1. Tentacle length (TNL). From the opening of the tentacle sheath. (1) Less than 3 times, (2) more than 3 times, or (3) more than 5 times the body length [19,23].
2. Tentilla length (TTL). Secondary tentacles or branches. (1) Less than half or (2) more than half the body length [22].
3. Tentilla coloration (TTC). Observable pigmentation of colloblasts while tentilla are contracted. (1) Yellow or (2) whitish [10,23] (Figures 1 and 2).
4. Gastrovascular cavity coloration (GCL). Pigmentation observable under the infundibulum. (0) Absent or (1) present (Figures 1 and 2).
5. Comb row proportion (CRP). In the tentacular plane. (1) Less than $3/4$ or (2) more than $3/4$ the body circumference [10,23] (Figures 1 and 2).
6. Comb row length (CRL). In every plane, all comb rows. (1) Same or (2) different size [10,23] (Figures 1 and 2).
7. Tentacle sheath spacing (SSP). Position of the tentacle sheath. (1) Next to or (2) far from the oral/aboral axis [17,19] (Figures 1 and 2).
8. Tentacle sheath shape (SSH). In the tentacular plane. (1) Spherical or (2) elongated (Figures 1 and 2).
9. Tentacle sheath opening (SOP). In the tentacular plane. (1) Under or (2) at the beginning of the comb rows (Figures 1 and 2).
10. Tentacle sheath angle (SAN). In the tentacular plane, from tentacular canal attachment and the horizontal. (1) Less than 45° or (2) more than 45° [17] (Figures 1 and 2).
11. Tentacle sheath location (SLC). Observable in the tentacular plane. (1) Under, (2) above, or (3) at the stomach level (Figures 1 and 2).
12. Tentacle sheath exit (SXT). Observable in the tentacular plane, referring to its shape or general appearance. (1) Straight or (2) oblique [17] (Figures 1 and 2).
13. Gastrovascular cavity proportion (GPT). From the mouth to the infundibulum. (1) Less than half or (2) more than half the body length [17,23] (Figures 1 and 2).
14. Gastrovascular cavity pigmentation pattern (GPP). Observable in the esophageal plane. (0) Absent or (1) present (Figures 1 and 2).
15. Gastrovascular cavity pigmentation pattern color (GPC). Observable in the esophageal plane. (1) Brown and yellow, (2) yellow, or (3) without color (Figures 1 and 2).
16. Stomach tissue margin (STM). Observable in the tentacular plane. (0) Absent or (1) present (Figure 2).
17. Stomach shape (STS). In the tentacular plane, without considering the connecting canals. (1) Star shape or (2) a different shape (Figures 1 and 2).
18. Infundibulum location (IFL). In the tentacular plane. (1) under half or (2) above half the body length (Figures 1 and 2).
19. Tentacular canal shape (TCS). Observable in the tentacular plane, referring to its shape or general appearance. (1) Straight or (2) oblique (Figures 1 and 2).

20. Tentacular canal direction (TCD). Observable in the tentacular plane, referring to its location. (1) Aboral region or (2) oral region of the body (Figures 1 and 2).
21. Adradial canal length (ADL). Observable in aboral view, from the interradial canal attachment. (1) Symmetrical or (2) asymmetrical (Figure 1).
22. Adradial canal attachment (ADA). Observable in the esophageal plane, to the meridional canals or comb rows. (1) Aboral region or (2) oral region of the body (Figure 1).
23. Aboral canal shape (ABS). In the tentacular plane, from the stomach attachment. (1) Straight or (2) spiral shape (Figures 1 and 2).
24. Pharyngeal fold size (PFS). Observable in the oral view and tentacular plane. (1) Conspicuous or (2) inconspicuous [17] (Figures 1 and 2).

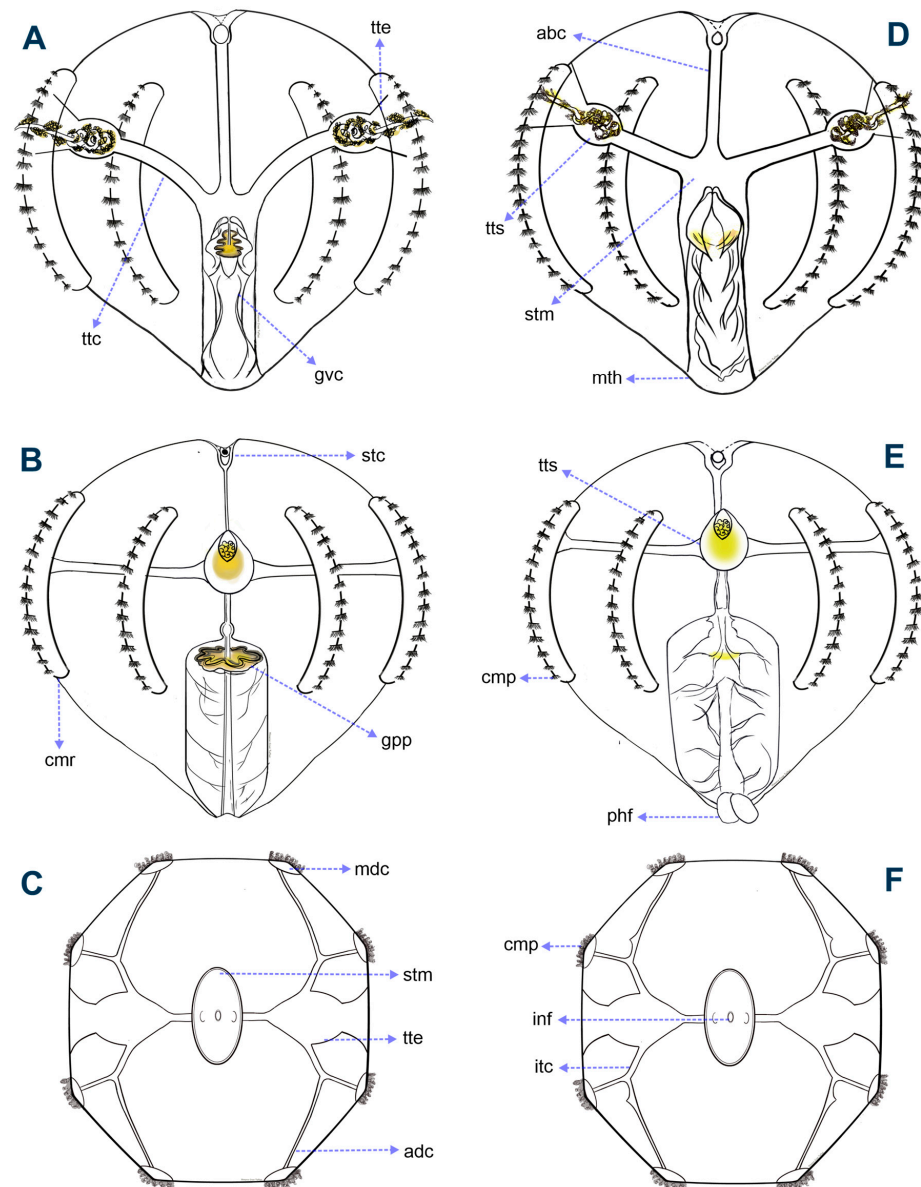


Figure 1. Schemes and principal morphological features of Mexican *Pleurobrachia* individuals observed in the Southern Mexican Pacific coast (POcM) region (A–C) and in the Gulf of Mexico (GoM) region (D–F). Side tentacular view (A,D). Side esophageal view (B,E). Aboral view (C,F). Abbreviations: abc, aboral canal; adc, adradial canal; cmp, comb plate; cmr, comb row; gpp, gastrovascular pigmentation pattern; gvc, gastrovascular cavity; inf, infundibulum; itc, interradial canal; mdc, meridional canal; mth, mouth; phf, pharyngeal folds; stc, statocyst; stm, stomach; ttc, tentacle canal; tte, tentacle sheath exit; and tts, tentacle sheath.

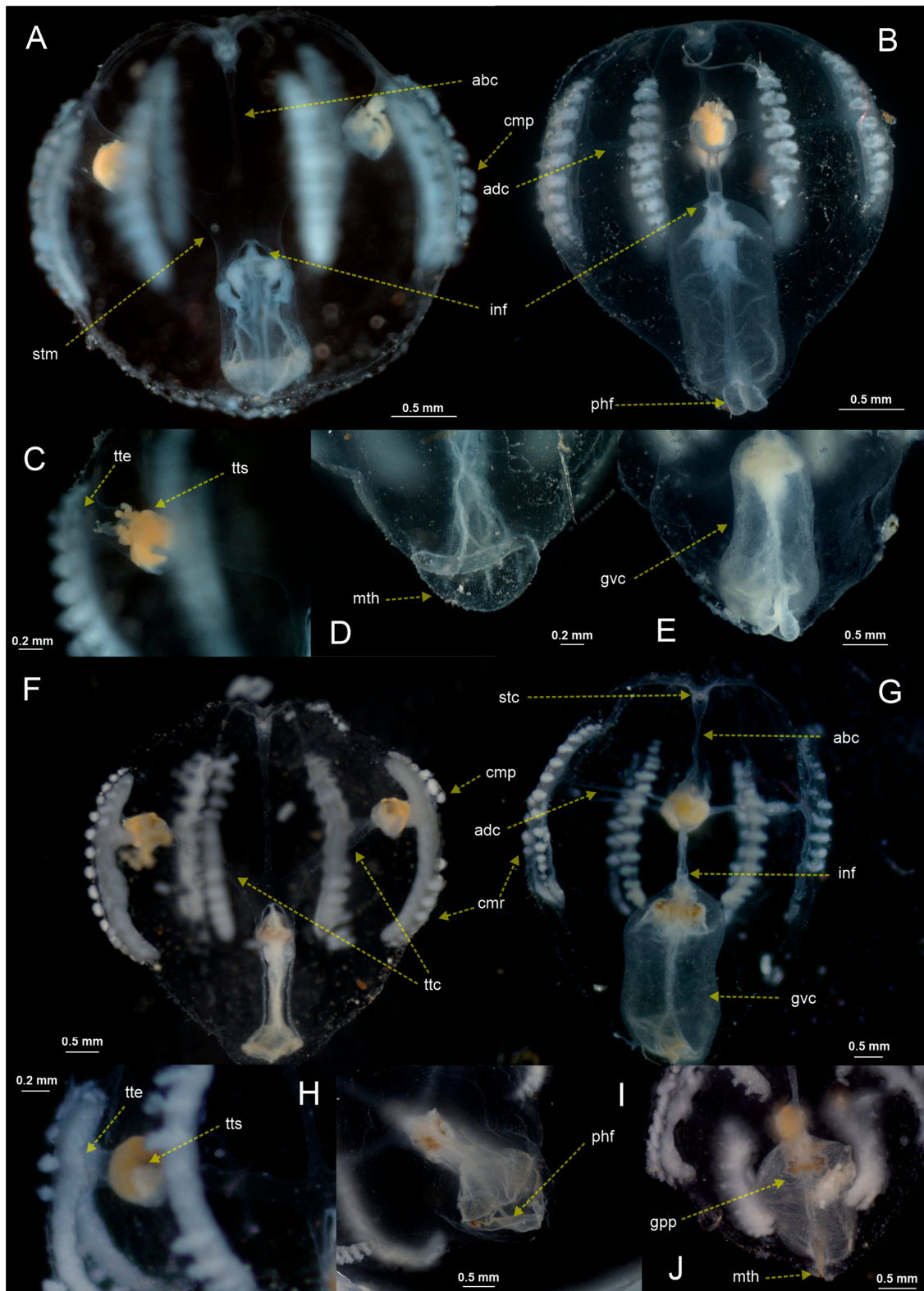


Figure 2. Discrete morphological attributes showing the distinctive character states of the individuals analyzed in the Gulf of Mexico (GoM) region (A–E) and in the Southern Mexican Pacific coast (POcM) region (F–J). Side tentacular view (A,F). Side esophageal view (B,G). Zoom to the tentacle sheath in the side tentacular view (C,H). Zoom to the gastrovascular cavity in the side tentacular view (D,E,I,J). Abbreviations: abc, aboral canal; adc, adradial canal; cmp, comb plate; cmr, comb row; gpp, gastrovascular pigmentation pattern; gvc, gastrovascular cavity; inf, infundibulum; mth, mouth; phf, pharyngeal folds; stc, statocyst; stm, stomach; ttc, tentacle canal; tte, tentacle sheath exit; and tts, tentacle sheath.

Due to the difficulty of preserving the specimens, quantitative attributes had not been considered by previous authors to identify differences within the species of the genus *Pleurobrachia* [10,17,19,22,23]. Here, we applied 14 continuous measurements, of which attributes 25 and 26 have already been used in other studies [45,60], while the remainder 27–38 are proposed in this study as follows (Figure 3). These characters are as follows:

25. Total body length (TBL). From the statocyst to the mouth opening, in the tentacular plane [45,60] (Figure 3).
26. Total body width (BWT). At the middle of the stomach, in the tentacular plane [45,60] (Figure 3).
27. Total body width (BWE). At the middle of the stomach, in the esophageal plane (Figure 3).
28. Gastrovascular cavity length (GVL). From the infundibulum to the mouth opening, in the tentacular plane (Figure 3).
29. Gastrovascular cavity width (GVW). At the widest region, in the tentacular plane (Figure 3).
30. Tentacular canal length (TCL). From the middle of the stomach to the tentacular sheath base, in the tentacular plane (Figure 3).
31. Aboral canal length (ACL). From the middle of the stomach to the statocyst base, in the tentacular plane (Figure 3).
32. Horizontal distance between tentacle sheaths (TSD). At the tentacular sheath middle level, in the tentacular plane (Figure 3).
33. Tentacle sheath length (TSL). From the sheath base to the end, in the tentacular plane (Figure 3).
34. Tentacle sheath width (TSW). At the widest region, in the tentacular plane (Figure 3).
35. Comb row circumference (CRC). Along lateral comb rows, measured by one side and duplicated for symmetry, in the tentacular plane (Figure 3).
36. Total body circumference (TBC). From the statocyst to the pharyngeal fold, measured by one side and duplicated for symmetry, in the tentacular plane (Figure 3).
37. Angle between tentacular canals (TCA). At the middle of the stomach, observable in the tentacular plane (Figure 3).
38. Tentacular canal angle (ATC). At the middle of the stomach, from the horizontal to the tentacular canal, observable in the tentacular plane (Figure 3).

The frequency of character states was examined for each discrete morphological character. Based on the frequency calculated for each locality, we built a contingency table to evaluate if the differences in frequency among character states were associated with different localities or regions. Contingency tables were analyzed with the chi-square test and the contingency coefficient [74]. The normality of the distribution of the quantitative continuous features was tested by the Shapiro–Wilk test, in the case of non-normal distribution, bias was shown. For all variables, the mean and standard deviation were calculated to determine whether the measurements differed between locations, where a one-way analysis of variance (ANOVA) and a Kruskal–Wallis test were performed according to the distribution of the variables [74].

To evaluate whether the phenotypic variation in Mexican *Pleurobrachia* supports the existence of at least two distinct morphological species as previously suggested [60–64], we conducted a series of multivariate ordination analyses. First, we conducted a principal components analysis (PCA) using pairwise covariance matrices to explore geographic patterns of variation among specimens based on the continuous characters. To approximate a classification and reduce the dimensionality of the analyzed features, a linear discriminant analysis (LDA) was performed with the same attributes. Then, a principal coordinates analysis (PCoA) was performed using a Gower pairwise distance matrix based

on 120 specimens and a combination of 24 discrete and 14 continuous morphological traits. Generalized linear models (GLMs) were performed to determine if the size of the organisms is related to latitude or to the marine regions. Body size was analyzed using the PC1 from the analysis of continuous attributes described above. Due to the type of morphometric datasets (especially with linear measurements), it is established that the greatest source of variation is quantified by a new variable PC1, often interpreted as a general representation of body size [75].

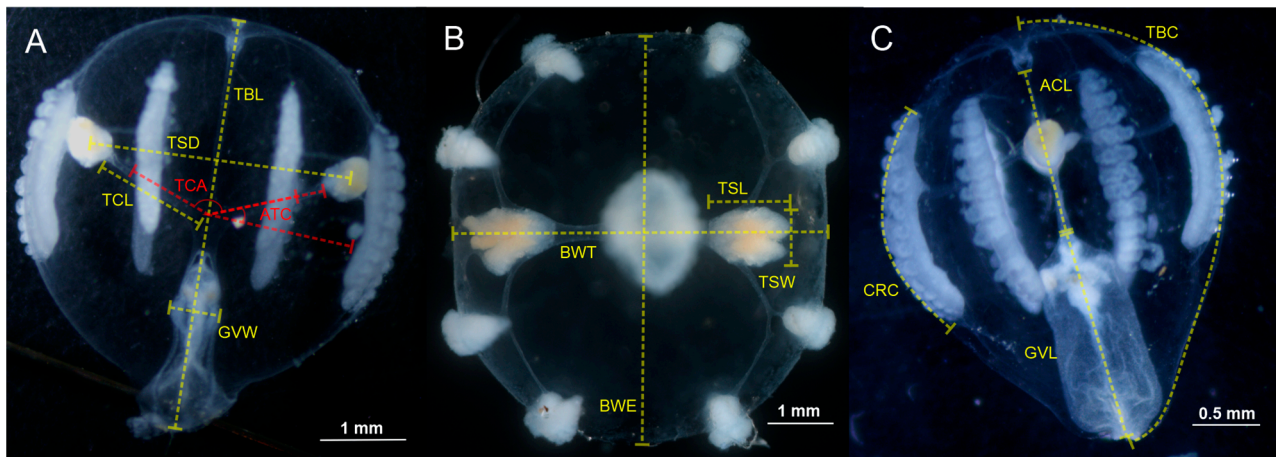


Figure 3. Measurements of continuous quantitative attributes regarding the internal and external morphological structures of *Pleurobrachia* specimens distributed along the coast of Mexico. Tentacular plane (A). Oral plane (B). Esophageal side plane (C). Abbreviations: ACL, aboral canal length; ATC, tentacular canal angle; BWE, total body width in the esophageal plane; BWT, total body width in the tentacular plane; CRC, comb row circumference; GVL, gastrovascular cavity length; GVW, gastrovascular cavity width; TBC, total body circumference; TBL, total body length; TCA, angle between tentacular canals; TCL, tentacular canal length; TSD, horizontal distance between tentacle sheaths; TSL, tentacle sheath length; and TSW, tentacle sheath width.

Given that taxonomic assignment of *Pleurobrachia* in Mexico has historically relied on geographic criteria, we conducted an additional analysis to test whether the observed morphological variation among Mexican specimens corresponds to that of *P. pileus* and *P. bachei* [60–64], as previously registered. For this purpose, we characterized the reference species using our discrete character set in conjunction with their seminal species descriptions [11,19,33]. The resulting coded matrices for *P. pileus* and *P. bachei* were analyzed alongside the matrix of 120 Mexican specimens using a non-metric multidimensional scaling (NMDS). Lastly, to observe the clustering tendency of operational taxonomic units (OTUs), a cluster analysis was performed using the same database with a bootstrap of 500 replicates, a Gower similarity index, and the UPGMA algorithm. Multivariate analyses were performed considering each specimen as an OTU. For all analyses, the alpha significance level was 0.05. All analyses were performed in PAST 4.03 [76].

3. Results

3.1. Discrete Morphological Characters

Of the 24 discrete morphological attributes analyzed, 15 showed the same character state in all individuals observed (Table S1). Meanwhile, the other six related to the tentacular sheath, tentacular canals, gastrovascular cavity (GVC), mouth, and infundibulum presented unique and consistent character states among all analyzed individuals from the Gulf of Mexico (GoM) localities: SOP tentacle sheath opening, SXT tentacle sheath exits, GPP pigmentation pattern of the GVC, GPC pigmentation pattern color of the GVC, TCS

tentacular canal shape, and PFS pharyngeal fold size, and these same attributes showed a unique and consistent alternative state in the Southern Mexican Pacific coast (POcM) region locality. The attributes GPT proportion of the GVC and STM stomach tissue margin showed different character states indistinctly in the different specimens analyzed from all localities. Finally, the ABS aboral canal shape attribute presented one character state in all GoM localities, while the POcM locality presented both character states, although the state observed in the GoM localities predominated (Table 2) (Figure 4).

Table 2. Frequencies of multistate or binary characters used to differentiate *Pleurobrachia* specimens among Mexican localities. Abbreviations: ABS, aboral canal shape; GPC, pigmentation pattern color of the GVC; GPP, pigmentation pattern of the GVC; GPT, proportion of the GVC; PFS, pharyngeal fold size; SOP, tentacle sheath opening; STM, stomach tissue margin; SXT, tentacle sheath exit; and TCS, tentacular canal shape.

Attribute	Character States	THVZ (%)	TCVZ (%)	BZVZ (%)	MTCH (%)	Chi ²	CC **
9.- SOP	(1) Under ^a	0	0	0	100	400 *	0.71
	(2) Beginning ^a	100	100	100	0		
12.- SXT	(1) Straight	100	100	100	0	400 *	0.71
	(2) Oblique	0	0	0	100		
13.- GPT	(1) Less than half ^b	50	53	90	63	43.14 *	0.31
	(2) More than half ^b	50	47	10	37		
14.- GPP	(0) Absent	100	100	100	0	400 *	0.71
	(1) Present	0	0	0	100		
15.- GPC	(1) Brown and yellow	0	0	0	100	400 *	0.71
	(2) Yellow	100	100	100	0		
	(3) Without color	0	0	0	0		
16.- STM	(0) Absent	47	100	20	63	137.21 *	0.51
	(1) Present	53	0	80	37		
19.- TCS	(1) Straight	100	100	100	0	400 *	0.71
	(2) Oblique	0	0	0	100		
23.- ABS	(1) Straight	100	100	100	80	63.16 *	0.37
	(2) Spiral	0	0	0	20		
24.- PFS	(1) Conspicuous	100	100	100	0	400 *	0.71
	(2) Inconspicuous	0	0	0	100		

** CC: contingency coefficient values. * $p \leq 0.001$: the significance of association of frequency between character states and localities. Superscripts on character states refer to the following: a = the position of the comb rows, and b = the total body length from the statocyst to the mouth.

According to the above, all specimens of Mexican *Pleurobrachia* presented the following: a tentacle length (TBL) 3 times larger than the total body length; a tentilla length (TTL) less than the half of the total body length; a tentilla yellow coloration (TTC): GVC coloration (GCL) present; a comb row proportion (CRP) less than three-quarters of the total body circumference; the same comb row length (CRL); tentacle sheath spacing (SSP) far from the oral/aboral axis; a spherical tentacle sheath shape (SSH); a tentacle sheath angle (SAN) less than 45°; a tentacle sheath location (SLC) above the stomach; a star-shaped stomach (STS); an infundibulum location (IFL) under the half of the total body length; a tentacular canal direction (TCD) in the aboral region; an asymmetrical adradial canal length (ADL), and adradial canal attachment (ADA) in the aboral region. Meanwhile, the differences in the other attributes could be grouped into two morphotypes according to the region where they are distributed, which are as follows:

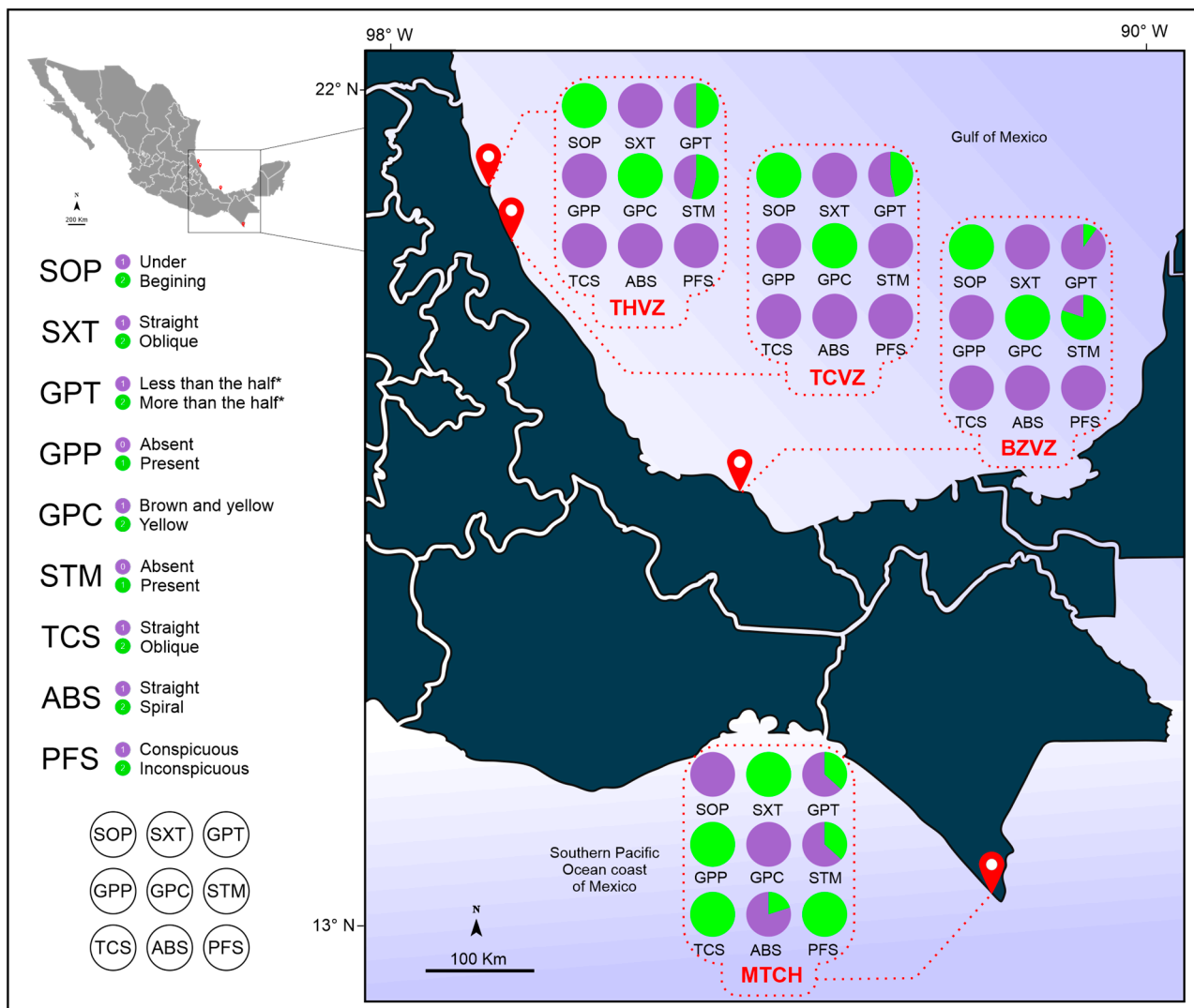


Figure 4. Distribution of distinctive morphological attributes of *Pleurobrachia* sp. from several locations in Mexican coast localities. Localities: THVZ, Tamiahua estuary; TCVZ, Tampamachoco lagoon; BZVZ, Balzapote bay; and MTCH, Madero port. Abbreviations: ABS, aboral canal shape; GPC, pigmentation pattern color of the GVC; GPP, pigmentation pattern of the GVC; GPT, proportion of the GVC; PFS, pharyngeal fold size; SOP, tentacle sheath opening; STM, stomach tissue margin; SXT, tentacle sheath exit; and TCS, tentacular canal shape.

GoM region: all specimens from localities THVZ, TCVZ, and BZVZ showed tentacular sheath opening (SOP) at the beginning of comb rows, a straight tentacular sheath exit (SXT), an absent GVC pigmentation pattern (GPP), a yellow GVC pigmentation (GPC), a straight tentacular canal shape (TCS), a straight aboral canal shape (ABS), and conspicuous pharyngeal folds (PFS); on the other hand, the proportion of the GVC (GPT) in most specimens was less than half of the body size, while the stomach tissue margin (STM) was mostly present in the THVZ and BZVZ localities and totally absent in TCVZ individuals (Table 2) (Figures 1, 2 and 4).

POcM region: all specimens from the MTCH locality showed a tentacular sheath opening (SOP) below the comb rows, an oblique tentacular sheath exit (SXT), a present GVC pigmentation pattern (GPP), a yellow and black GVC pigmentation (GPC), an oblique tentacular canal shape (TCS), and inconspicuous pharyngeal folds (PFS); on the other hand, the proportion of the GVC (GPT) in most specimens was less than half of the body size, the

margin of the stomach tissue (STM) was mostly absent, and the shape of the aboral canal (ABS) was mostly straight (Table 2) (Figures 1, 2 and 4).

3.2. Continuous Quantitative Morphological Characters

We observed a body clinal trend with respect to the size of the organisms and the latitude of the localities; the smallest specimens were in the northern localities and the largest corresponded to those from the southern locality (MTCH) (Table 3). Size differences among localities were supported by Kruskal–Wallis and ANOVA tests in the following attributes: BWE ($H = 70.01$, $p < 0.001$), ACL ($H = 68.60$, $p < 0.001$), and TBC ($H = 68.79$, $p < 0.001$) (Table 3). There were also significant differences between the THVZ and MTCH locations for the attributes TBL ($H = 64.15$, $p < 0.001$), BWT ($F = 47.96$, $p < 0.001$), GVL ($H = 57.93$, $p < 0.001$), and TSD ($F = 41.20$, $p < 0.001$), CRC ($H = 50.47$, $p < 0.001$). In addition, there were significant differences between groups of localities: THVZ and TCVZ were significantly different from BZVZ and MTCH for the attributes GVL ($F = 47.27$, $p < 0.001$), TSL ($F = 25.98$, $p < 0.001$), TSW ($F = 17.98$, $p < 0.001$), and ATC ($F = 8.23$, $p < 0.001$). Finally, only one attribute showed no significant differences among all specimens of the analyzed locations: TCA ($H = 7.60$, $p > 0.05$) (Table 3).

Table 3. Measurements of continuous quantitative morphological attributes as mean $\pm$ standard deviation (min, max) analyzed to evaluate differences in *Pleurobrachia* specimens among Mexican localities. Abbreviations: ACL, aboral canal length; ATC, tentacular canal angle; BWE, total body width in the esophageal plane; BWT, total body width in the tentacular plane; CRC, comb row circumference; GVL, gastrovascular cavity length; GVL, gastrovascular cavity width; TBC, total body circumference; TBL, total body length; TCA, angle between tentacular canals; TCL, tentacular canal length; TSD, horizontal distance between tentacle sheaths; TSL, tentacle sheath length; and TSW, tentacle sheath width.

Keys	THVZ	TCVZ	BZVZ	MTCH	F/H
TBL ^l	2.45 ^b $\pm$ 0.12 (1.34, 3.74)	3.78 ^a $\pm$ 0.16 (2.12, 5.76)	4.34 ^a $\pm$ 0.22 (2.28, 6.70)	5.48 ^c $\pm$ 0.24 (2.12, 8.64)	64.15 ^{H**}
BWT ⁿ	2.20 ^b $\pm$ 0.12 (0.82, 3.49)	3.28 ^a $\pm$ 0.12 (2.2, 4.68)	3.86 ^a $\pm$ 0.19 (2.01, 6.00)	4.84 ^c $\pm$ 0.19 (2.02, 7.22)	47.96 ^{F**}
BWE ^l	2.13 ^a $\pm$ 0.09 (1.27, 3.29)	3.06 ^b $\pm$ 0.10 (2.09, 4.03)	3.60 ^c $\pm$ 0.18 (1.95, 5.69)	4.58 ^d $\pm$ 0.17 (2.27, 7.01)	70.10 ^{H**}
GVL ^l	1.20 ^b $\pm$ 0.07 (0.64, 1.87)	1.91 ^a $\pm$ 0.09 (1.1, 3.15)	2.00 ^a $\pm$ 0.11 (1.01, 2.99)	2.72 ^c $\pm$ 0.14 (1.02, 4.71)	57.93 ^{H**}
GVW ⁿ	0.47 ^a $\pm$ 0.02 (0.22, 0.75)	0.47 ^a $\pm$ 0.02 (0.29, 0.7)	0.69 ^b $\pm$ 0.02 (0.48, 0.94)	0.77 ^b $\pm$ 0.02 (0.55, 1.06)	47.27 ^{F**}
TCL ⁿ	0.41 ^c $\pm$ 0.04 (0.11, 0.88)	0.91 ^a $\pm$ 0.04 (0.44, 1.44)	1.10 ^{a,b} $\pm$ 0.07 (0.35, 1.89)	1.22 ^b $\pm$ 0.08 (0.22, 2.14)	37.16 ^{F**}
ACL ^l	0.71 ^a $\pm$ 0.04 (0.3, 1.16)	1.25 ^b $\pm$ 0.06 (0.61, 1.91)	1.57 ^c $\pm$ 0.09 (0.71, 2.47)	1.91 ^d $\pm$ 0.10 (0.60, 2.95)	68.60 ^{H**}
TSD ⁿ	1.24 ^b $\pm$ 0.08 (0.55, 2.13)	2.12 ^a $\pm$ 0.09 (1.14, 3.19)	2.50 ^a $\pm$ 0.13 (1.21, 4.11)	2.93 ^c $\pm$ 0.14 (1.00, 4.79)	41.20 ^{F**}
TSL ⁿ	0.48 ^a $\pm$ 0.02 (0.27, 0.68)	0.46 ^a $\pm$ 0.02 (0.21, 0.82)	0.63 ^b $\pm$ 0.02 (0.39, 0.88)	0.69 ^b $\pm$ 0.02 (0.46, 0.93)	25.98 ^{F**}
TSW ⁿ	0.39 ^a $\pm$ 0.02 (0.18, 0.59)	0.36 ^a $\pm$ 0.02 (0.15, 0.52)	0.47 ^b $\pm$ 0.02 (0.35, 0.64)	0.53 ^b $\pm$ 0.02 (0.31, 0.86)	17.98 ^{F**}
CRC ^l	2.85 ^b $\pm$ 0.17 (1.44, 4.8)	4.13 ^a $\pm$ 0.18 (2.46, 6.54)	4.45 ^a $\pm$ 0.26 (2.16, 7.08)	5.87 ^c $\pm$ 0.28 (2.68, 8.90)	50.47 ^{H**}
TBC ^l	7.69 ^a $\pm$ 0.39 (4.2, 11.92)	11.28 ^b $\pm$ 0.45 (6.62, 17.06)	13.28 ^c $\pm$ 0.65 (7.40, 20.02)	16.92 ^d $\pm$ 0.70 (7.34, 26.56)	68.79 ^{H**}
TCA ^l	21.97 ^a $\pm$ 0.91 (8.33, 30.84)	20.73 ^a $\pm$ 1.27 (4.55, 30.23)	25.01 ^a $\pm$ 0.97 (14.02, 35.9)	25.30 ^a $\pm$ 1.27 (11.42, 36.57)	7.60 ^{H*}
ATC ⁿ	136.42 ^a $\pm$ 1.49 (118.56, 153.7)	137.81 ^a $\pm$ 1.79 (122.97, 145.69)	128.18 ^b $\pm$ 1.60 (111.61, 145.69)	128.89 ^b $\pm$ 2.03 (109.22, 149.75)	8.23 ^{F**}

** $p < 0.001$, * $p < 0.05$: values of significance of the applied tests among localities. F superscripts show analysis of variance (ANOVA) test values; H super index shows Kruskal–Wallis test values. Key superscript index means normality or direction of bias on variable distribution, n = normal distribution, l = left bias. Superscript letters above the mean values refer to the differences among means, where the same letter refers to no significant difference among those locations and different letters show a significant difference.

3.3. Multivariate Analyses

The principal components analysis (PCA) corresponding to the fourteen continuous quantitative attributes, including specimens from four localities, explained more than 98% of the total variation in the first two principal components (PC1 = 89.63%, PC2 = 9.04%). The operational taxonomic units (OTU's) were partially segregated in the multivariate space of the first two PC's. The linear discriminant analysis, LDA, also explained 86.89% of the variation in the first two axes (Axis1 = 57.4%, Axis2 = 29.49%) (Figure 5A). The first two principal coordinates (PCoA) of the continuous and discrete attributes explained 59.68% of the variations (PCo1 = 48.54%, PCo2 = 11.14%). The respective scatter plot showed that individuals from the GoM and POcM marine regions reside in discrete phenotypic groups

in the multivariate space analysis, where north localities of the GoM (THVZ = 93.33%, TCVZ = 93.33%) had a higher percentage of self-assignment than those in the south of the GoM (BZVZ = 76.67%) and in the POcM region (MTCH = 80.00%) in the confusion matrix, although the value of $\chi^2 = 74$ evidenced statistically significant differences (Figure 5B). Regarding the generalized linear models (GLM's), the first one (Figure 5C) showed a deviance of 47.23%, while the second one (Figure 5D) presented a higher deviance of 64.31%, indicating a greater trend in differences in the size of the organisms with respect to the marine regions in which the sampled specimens are located than between localities analyzed independently.

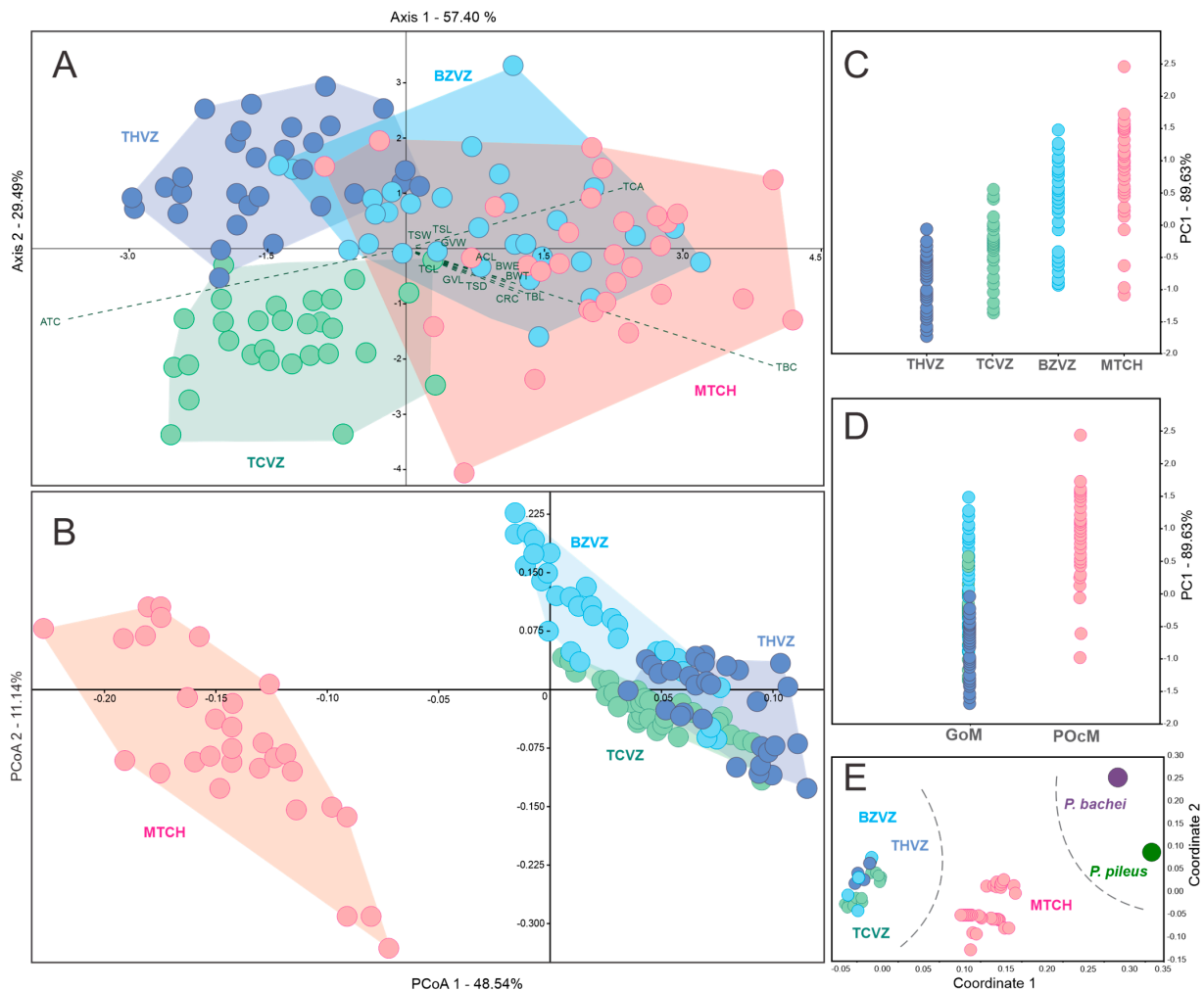


Figure 5. Scatter plots of ordination analyses to evaluate the morphological variation in Mexican *Pleurobrachia* specimens. Linear discriminant analysis (LDA) using 14 continuous quantitative characters, (A). Principal coordinate analysis (PCoA) using the 24 discrete and 14 continuous features of all specimens (B). Generalized linear model (GLM) using principal component 1 (PC1) vs. localities (C) and generalized linear model using PC1 vs. regions (D). Non-metric multidimensional scaling (NMDS) using only the 24 discrete attributes and adding the species *P. pileus* and *P. bachei* (E). In the center of the scatter plot, vectors correspond to the contribution of the traits in the multivariate space (A). Abbreviations: GoM, Gulf of Mexico; POcM, Southern Mexican Pacific coast; THVZ, Tamiahua estuary; TCVZ, Tampamachoco lagoon; BZVZ, Balzapote bay; MTCH, Madero port; ACL, aboral canal length; ATC, tentacular canal angle; BWE, total body width in the esophageal plane; BWT, total body width in the tentacular plane; CRC, comb row circumference; GVL, gastrovascular cavity length; GVV, gastrovascular cavity width; TBC, total body circumference; TBL, total body length; TCA, angle between tentacular canals; TCL, tentacular canal length; TSD, horizontal distance between tentacle sheaths; TSL, tentacle sheath length; and TSW, tentacle sheath width.

The non-metric multidimensional scaling (NMDS) based on the matrix of all discrete attributes allowed us to identify two clearly differentiated groups within the *Pleurobrachia* specimens collected in Mexican waters. Equally, this analysis evidenced the distant location in multivariate space of the morphotypes of the species *P. pileus* and *P. bachei* (Figure 5E). The resulting dendrogram of the clustering analysis showed two main clades with high statistical support, corresponding to specimens from different oceanic regions, which were different from the previously recorded species (Figure 6). A first clade, identified as the “Southern Pacific Ocean coast of Mexico Cluster,” grouped specimens collected on the southern coast of the Mexican Pacific (MTCH). A second clade corresponded to the “Gulf of Mexico Cluster” and grouped specimens collected in THVZ, TCVZ, and BZVZ. These two clades were supported as sister groups with 99% support, and they were also associated with 100% support to a basal clade comprising both *P. pileus* and *P. bachei*. These latter species were associated with 96% support, marking a considerable distance from the Mexican specimens (CCC = 0.99) (Figure 6).

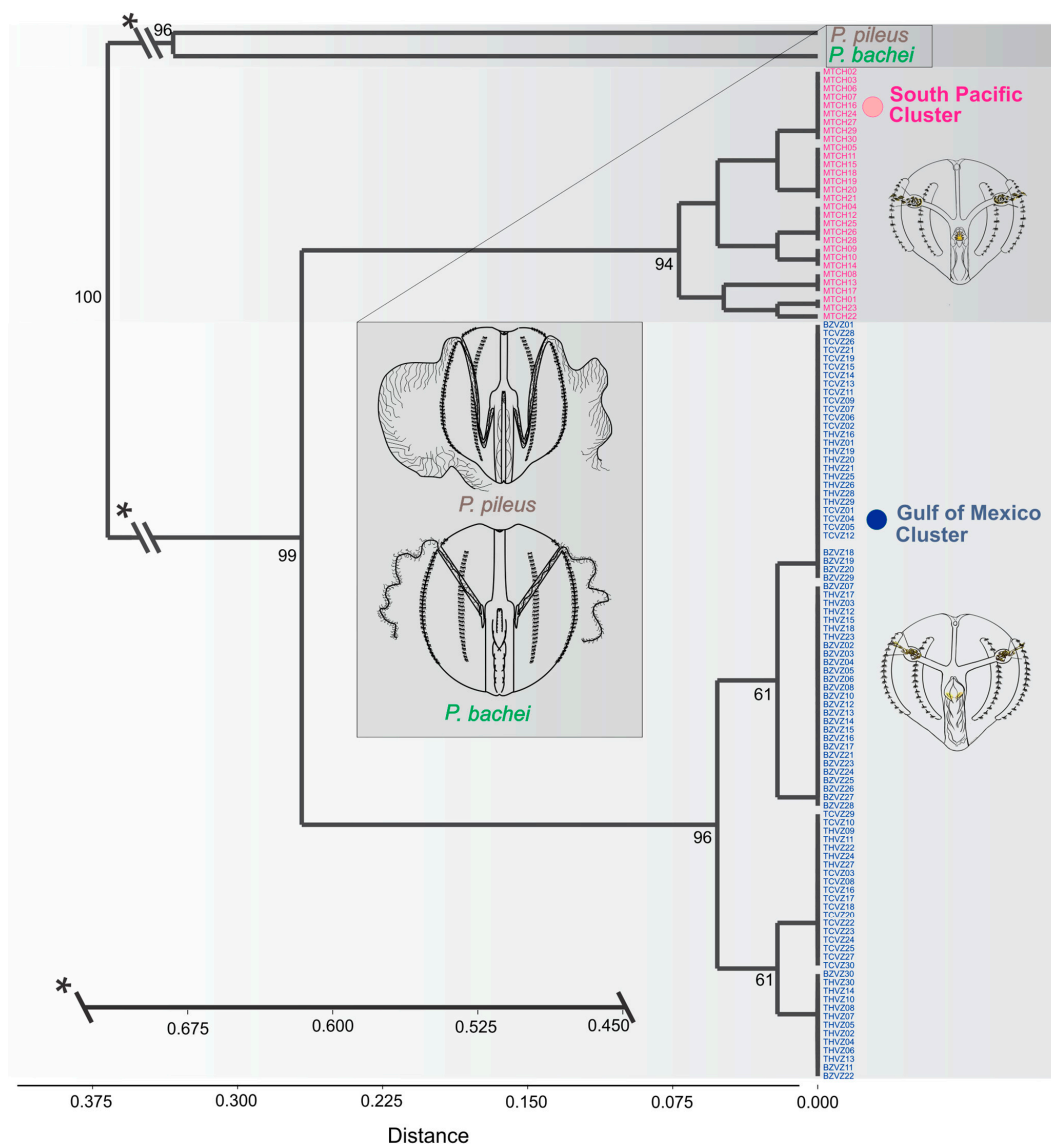


Figure 6. Cluster by distance using the 24 discrete attributes of all Mexican *Pleurobrachia* specimens and adding the species *P. pileus* and *P. bachei* with a bootstrap of 500 replicates (only bootstrap values of nodes above 50% are shown). Abbreviations: THVZ, specimens from Tamiahua estuary; TCVZ, specimens from Tampamachoco lagoon; BZVZ, specimens from Balzapote bay; and MTCH, specimens from Madero port. *Axis brake.

4. Discussion

The taxonomy of the genus *Pleurobrachia* in Mexico is a clear example of the global challenges facing the systematics of ctenophores [12,13,51,77]. Existing records of *P. pileus* in the Gulf of Mexico and *P. bachei* in the Pacific are well documented but are based predominantly on geographic criteria [73], following the seminal descriptions of both species [72,78]. However, the results of this study suggest that the Mexican populations attributed to *P. pileus* and *P. bachei* may in fact represent distinct species that have not yet been formally described. The presence of clearly defined regional morphological patterns and the lack of overlap with traditional diagnostic traits demonstrate the need to reconsider the taxonomy of these organisms in the region. Our findings open the door to future research integrating morphological, genetic, and ecological data, which are essential for accurately delimiting species boundaries and obtaining a better understanding of the biodiversity and biogeography of ctenophores, as has been developed in other organisms [79–82]. Based on Darwin’s morphological species criterion [83], this study proposes the existence of two distinct morphological species of *Pleurobrachia*. The recognition of these two entities is based on an analysis of key morphological structures that were already used in the definition of species of the genus, which validates the proposed classification [84].

Over the last two centuries, *Pleurobrachia* species have been described based on morphological features such as the position and shape of the tentacular sac, the tentacular sheath exit, the proportion of the gastrovascular cavity, and the pigmentation of the tentacles [10,17,19,22,23]. However, the most recent records and studies of the most studied species, *P. pileus* and *P. bachei*, have focused on physiological, ecological, and evolutionary aspects, omitting crucial details about the morphology of the species in the regions where they were studied [30,40,42,43,45–47,53]. This has led to their identification in Mexico using outdated descriptions [72,78].

Furthermore, the taxonomy of ctenophores is notoriously difficult due to the extremely fragile nature of these organisms; specimens are easily damaged during collection, and their gelatinous bodies are poorly preserved in traditional fixatives such as formaldehyde. This problem not only hides detailed morphological analysis, but also degrades DNA, complicating molecular studies [12,15,34]. As a result, in Mexico, many database records or zooplankton studies have only identified “*Pleurobrachia* sp.” [65–71], underestimating the true species richness and making it difficult to trace the biogeography of the group. In our study, the incorporation of new handling and fixation techniques was essential to properly identify morphological attributes and outperform traditional observations, which on several occasions has proven to be insufficient for clear identification of species in this genus [51,66,77].

We found solid evidence for the existence of two well-defined morphotypes. One of them, the Gulf of Mexico (GoM) morphotype, differs from *P. pileus* in several diagnostic characteristics: the latter has elongated and curved tentacular sheaths located below the stomach; the sheath exits are curved and open to the outside below the end of the comb rows in the aboral region; the stomach is reduced and elongated towards the pharyngeal canals; the GVC covers more than half the body length; the pharyngeal folds are short; and the tentacles have longer tentilla than the body length [10,11,17,19,21] (Figures 5E and 6).

Conversely, the Southern Mexican Pacific coast (POcM) morphotype differs from *P. bachei* in that this species has elongated tentacular sheaths located laterally at the level of the infundibulum below the stomach; the sheath opening is curved towards the interior of the body but has a straight exit below the end of the comb rows in the aboral region; the stomach is small and elongated towards the pharyngeal canals and the aboral region; the GVC is thin and covers more than half of the total body length; the pharyngeal folds and

mouth are reduced and not very prominent; and the tentacles have short tentilla less than half the body length [11,16,19,23] (Figures 5E and 6).

The morphological evidence obtained and compared supports the idea that the specimens found in the GoM and POcM regions belong to two unique morphotypes with evident and diagnosable characteristics. No correspondence was found with the descriptions of the species previously assigned as *P. pileus* and *P. bachei* [10,11,16,17,19,21–23] (Figures 5E and 6).

Statistical analysis of morphometric variation showed a clinal trend in organism size, with smaller sizes in northern locations and larger sizes in southern locations, regardless of marine region. This trend differs from Bergmann's rule [85–87], highlighting that size, a plastic trait, is not a reliable character for identification to the species level in this genus. The importance of assessing geographic variation in attributes and determining discrete diagnostic characters that are not susceptible to eco-geographic variation is perfectly illustrated by our approach. Changes in size and coloration often reflect phenotypic plasticity in response to environmental conditions and not necessarily reflect a difference at the species level, as has been showed in studies with other marine organisms [88–90].

Our study demonstrated the effectiveness of 24 discrete morphological attributes and showed that 9 of them (ABS, aboral canal shape; GPC, pigmentation pattern color of the GVC; GPP, pigmentation pattern of the GVC; GPT, proportion of the GVC; PFS, pharyngeal fold size; SOP, tentacle sheath opening; STM, stomach tissue margin; SXT, tentacle sheath exit; and TCS, tentacular canals shape) were adequate and consistent for identifying two morphotypes of *Pleurobrachia*. The anatomical structures that allowed these diagnostic differences to be established were the opening and exit of the tentacular sheath, the pattern and color of the GVC pigmentation, the shape of the tentacular canal, and the pharyngeal folds, among others (Figure 2). These findings demonstrate that combining discrete and continuous quantitative data provides a robust approximation of inter- and intraspecific differences [91].

The necessity to carry out modern and integrative taxonomic revisions is of utmost importance in this phylum. Currently, there are 185 species described [11], and it is estimated that there are around a similar number of species waiting to be discovered [2]. In addition, many of the current species have undergone various taxonomic reassignments [2,13]. Despite problems with the use of traditional taxonomy in these species, fossil ancestors of this group have been recognized by identifying unique sclerotized anatomical structures [92,93]. In the case of *Pleurobrachia* species, it is crucial to continue incorporating studies on ecological, ontogenetic, and molecular aspects, along with morphological studies (such as this one), to elucidate the boundaries between the eight currently recognized species and the two we propose.

5. Conclusions

This study achieved a promising approximation of the possible diversity of *Pleurobrachia* species by finding two new morphological species distributed along the coasts of Mexico. Previous records of *P. pileus* in the Gulf of Mexico and *P. bachei* in the Mexican Pacific Ocean, although well documented morphologically, should be considered provisional and await further analysis in more locations in Mexican marine regions. The geographical separation of these coasts suggests that the populations are distinct evolutionary lineages, a pattern of allopatric speciation observed in other marine organisms [94,95].

Consequently, the incorporation of molecular data from these Mexican populations would allow for the definitive validation of their identity and confirmation of their taxonomic status. The fragility and poor preservation of ctenophores have created a methodological barrier that has stopped significant progress in their systematics, perpetuating a

cycle in which both the lack of adequate morphological descriptions and the lack of genetic reference data hide the validation of records and naming of these species. Integrative taxonomy is the way to solve this problem and reveal the true species richness in the genus *Pleurobrachia*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d17100713/s1>, Supplementary Table S1. Frequencies of multistate or binary characters used to differentiate *Pleurobrachia* specimens among Mexican localities.

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Institutional Review Board Statement: This study complies with Mexican regulations regarding the ethical treatment of research subjects. The Dirección General de Vida Silvestre does not consider this species a priority, given its large population size, wide distribution in Mexico, and because it is not listed or included within any of the threat categories under NOM-059-SEMARNAT-2010.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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