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EVOLUTIONARY MORPHOLOGY OF *LIGOPHORUS* SPP. (MONOGENEA: DACTYLOGYRIDAE): A GEOMETRIC MORPHOMETRICS APPROACH

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D. Juan Antonio Balbuena Díaz-Pinés, Profesor Titular del Departamento de Zoología de la Facultad de Ciencias Biológicas de la Universidad de Valencia,

CERTIFICA que **Dª Abril Rodríguez González** ha realizado bajo mi dirección, y con el mayor aprovechamiento, el trabajo de investigación recogido en esta memoria, y que lleva por título "**EVOLUTIONARY MORPHOLOGY OF *LIGOPHORUS* SPP. (MONOGENEA: DACTYLOGYRIDAE): A GEOMETRIC MORPHOMETRICS APPROACH**", para optar al grado de Doctora en Ciencias Biológicas.

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Con amor a mis padres

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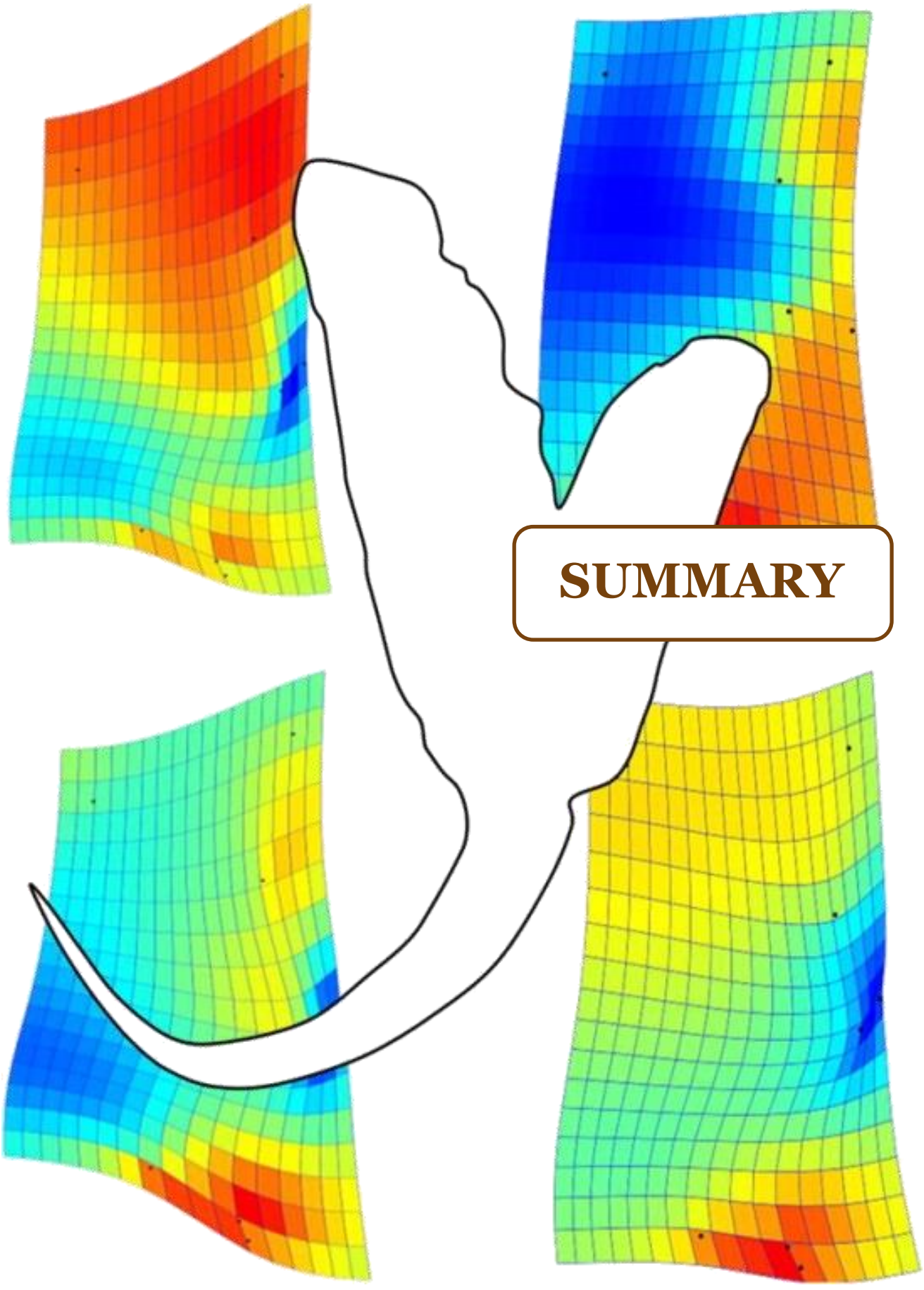


SUMMARY

Ligophorus spp. (Monogenea: Dactylogyridae) are common parasites of grey mullets from the Mediterranean Sea, the Black Sea and Sea of Azov (Sarabeev et al. 2013), which display remarkable morphological variation in their attachment organ. Thus, they offer an excellent model for investigating to which extent the phenotypic variability of the attachment organs among congeners is related to host specificity, environmental components, host-parasite coevolution and phylogeny (Vignon et al. 2011). In fact, monogeneans in general provide good models for studying morphological variation in attachment organs to test evolutionary constraints on shape (Šimková et al. 2001). Geometric morphometrics is currently considered as an ideal tool to tackle these problems (Slice, 2007), but it has been hardly applied to Monogenea and other parasites at large.

In this thesis, we developed a geometric morphometrics framework, combined with multivariate statistics, to explore intra e interspecific morphological variations in shape and size haptoral structures in 14 species of *Ligophorus* from 6 grey mullet species. In addition, we evaluated, in the context of phylogenetically independent contrasts (PIC), the evolutionary modularity and morphological integration between units in the haptor (i.e. roots and points) across species. The modules in the haptor structures were identified, providing first insight into how these structures are morphologically organized, how they vary and how they evolve. Consequently, we explored to which extend modular structure in the anchors of *Ligophorus* were accounted for adaptive and phylogenetic factors acting at different levels.

The following specific objectives were addressed:

1) To analyze the variability in shape and size of the dorsal and ventral anchors of *Ligophorus cephali* from *Mugil cephalus*, by means of geometric morphometrics and multivariate statistics, to assess the morphological integration between anchors and between roots and points.
2) To describe a new species of *Ligophorus* from *M. cephalus* from the Yucatán Peninsula, Mexico and to update the zoogeography of *Ligophorus* spp. in the light of current evidence for a complex of cryptic species.

3) To determine whether variation in the anchor shapes in 14 *Ligophorus* spp. is modular and integrated after evaluation of four hypotheses of modularity at both, adaptive and evolutionary levels.

4) To assess phylogenetic signal in form of ventral and dorsal anchors of 14 species of *Ligophorus* occurring on grey mullets from the Mediterranean, the Black Sea and Sea of Azov, to establish whether similarity in anchor form is explained by convergence or shared evolutionary history.

As a synthesis of this PhD study, the following findings and conclusions were drawn:

This study documented for first time the phenotypic plasticity and morphology of haptoral structures in *Ligophorus cephali* on *Mugil cephalus*. The pattern of shape variation observed was similar in ventral and dorsal anchors, with narrow and elongated anchors and short anchors. Interestingly, localised shape variation was much higher in the dorsal anchors, which is in line with the higher residual variation associated with dorsal anchors in the shape models. Moreover, in the size models the residual variation of the dorsal anchors was much higher than those of ventral anchors. In addition, we demonstrated that random effects (gill section × host individual) were an important determinant of shape in ventral, but no in dorsal anchors, and size models of dorsal and ventral anchors were clearly different. These differences between dorsal and ventral anchors in both shape and size perhaps reflect different functional roles in the attachment to the gills. In addition, the morphology in *Ligophorus* of haptoral structures dissimilar in shape and size revealed that curvature of the dorsal anchor can vary sharply, suggesting that the dorsal anchor/bar complex is more mobile than ventral one in this genus. This suggests a tighter control of the shape and size of ventral hanchors to fit the characteristics of the individual host microenvironment.

We observed high morphological integration in shape between ventral and dorsal anchors in *L. cephali* suggesting strong coordination in the parts of the haptoral structure which reveals that the shapes are not independent characters and could be driven by host specificity. In addition we

observed integration within parasite anchors (point and roots) in both anchors. This high level of morphological integration indicates a concerted action between anchors and suggests that a large fraction of the observed phenotypic variation does not compromise the functional role of anchors as levers.

We found that gill arch was an important determinant of anchor shape and size in *L. cephali*. The phenotypic plasticity in anchor morphology can reflect the ability of individuals of this species to colonise a new host, responding quickly to varying environmental conditions.

In order to address diversity within this genus in other geographical areas, a new species of *Ligophorus*, *Ligophorus yucatanensis*, from the gills of the flathead *M. cephalus* from México was described. This species was differenciated from all other species of *Ligophorus* by the morphology of the accessory piece of the copulatory organ. In addition the new species was distinguished by the morphology of the haptoral ventral bar and the distal end of the vaginal duct. In addition, the ventral anchors were shorter than those of all other species of *Ligophorus* reported in the Gulf of Mexico.

The new species resembles more closely species from the Mediterranean Sea and off the coast of the Northwestern Pacific than those recorded in South and North America. *Ligophorus yucatanensis* was included into Entity 4 (Western North Atlantic) according to zoographic records of *Ligophorus* spp. from the *M. cephalus* species complex.

We tested four different hypotheses of modularity in the haptoral anchors of 14 monogeneans species of *Ligophorus* evaluated by geometric morphometrics and phylogenetically independent contrasts (PIC). The roots and points represented two modules in the dorsal and ventral anchors, but modularity was not statistically supported when parasite phylogeny was accounted for, which indicated convergent evolution probably related to host characteristics and gill morphology. Moreover, PIC revealed medial and lateral modules in ventral anchors only. In contrast we found

evidence for ventral and dorsal anchors pairs forming two modules, supporting the notion that they play different functional roles. Integration between all identified modules was strong.

So, there is modular structure in the anchors of *Ligophorus* spp. accounted by adaptive and phylogenetic factors acting at different levels, and ventral and dorsal anchors evolve as integrated modules with specific roles in the attachment.

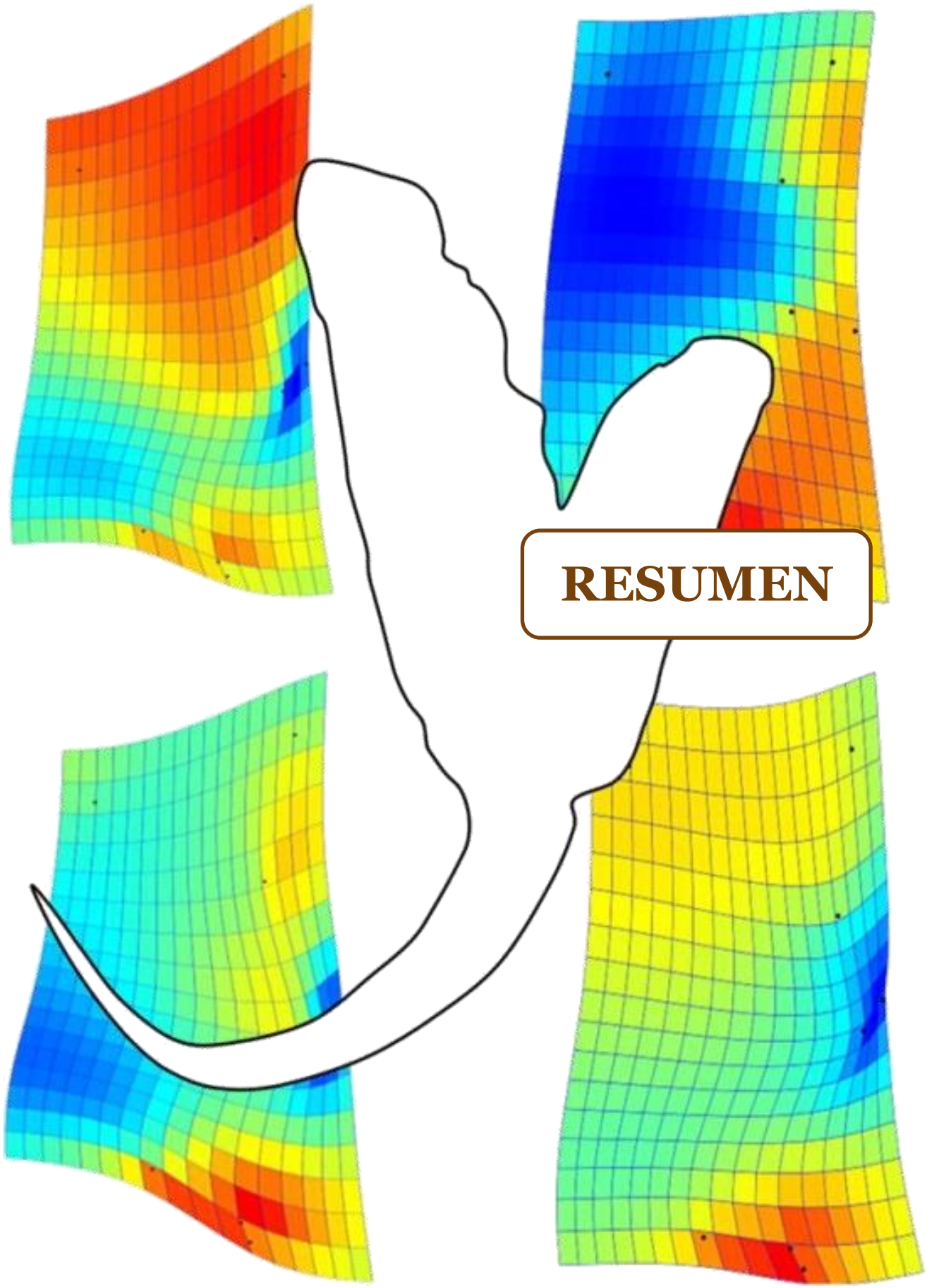
The patterns of morphological change in haptoral anchors were interpreted to reconstruct the dynamics of the evolutionary processes and visualized as paths from ancestors to descendants through the phylomorphospace. The tests performed for phylogenetic signal provided strong evidence for evolutionary processes playing a major role in determining the shape and, to a lesser degree, the size of the haptoral anchors.

The position in the phylomorphospace of distantly related species co-occurring on a given host species were very different: *L. confusus* and *L. imitans* parasitizing *Liza ramada* represent different clades and the anchors fell far apart in the shape and size morphospaces. *L. szidati* and *L. vanbenedenii* co-occurring on *Liza aurata* were placed in different clades, differed in shape and size of the dorsal anchors. So, no clear evidence for homoplasy in the *Ligophorus* spp. studied was found.

In several cases, members of clades that occur in the same host species showed similar anchor forms (*L. cephali*-*L. mediterraneus* on *M. cephalus*, *L. llewellyni* and *L. pilengas* on *Liza haematocheila*) or similar shapes (*L. acuminatus* and *L. minimus* on *Liza saliens*). These clades probably resulted from several intra-host duplication events and their morphological similarities point to occurrence of phylogenetic constrains on anchor form.

The phylogenetic position of *L. imitans* showed affinities with species found on *Lz. saliens*, suggesting that its occurrence on *Lz. ramada* represents a host-switch. The adaptation to a new host did not impose strong changes in haptoral anchor morphology and supports the notion of phylogeny being a major determinant of anchors morphology in *Ligophorus*. Thus, the variation of shape and size of ventral and dorsal anchors in 14 species of *Ligophorus* is largely determined by

common descent and shared evolutionary history. Although homoplasy dictated by adaptations to the host or to specific gill microhabitats could not be ruled out completely, its role seemed less important.



RESUMEN

Introducción general

La morfología es la ciencia de la forma, el estudio de cómo y porqué los organismos presentan una determinada apariencia. Un concepto central en la teoría evolutiva Darwiniana (Richter y Wirkner, 2013) y de la síntesis moderna, es que los caracteres específicos son el resultado de la adaptación y que dicha adaptación es forzada por la selección natural (Losos, 2011). Esto implica que las transformaciones evolutivas del fenotipo pueden ser explicadas en términos de una ventaja selectiva de una unidad evolutiva sobre otra, de la misma transformación en un ambiente dado (Richter y Wirkner, 2013). Por otro lado, D' Arcy Thompson en su libro *On Growth and Form* argumentó que las transformaciones evolutivas de la forma de los organismos pueden ser descritas por expresiones matemáticas basadas sobre fuerzas físicas que actúan sobre ellos. Así, los orígenes de la Morfometría Geométrica (MG) nacieron motivados en parte, por los estudios en grillas deformadas de Thompson (1942) en los años 80 (Polly et al. 2016).

El desarrollo de nuevas propiedades basadas en una teoría matemática coherente, capaz de capturar la forma, hace que esta nueva morfometría haya sido denominada como geométrica. Su recepción fue acogida como una "revolución" para el mundo del análisis morfológico, debido a la gran potencialidad y poder analítico de este nuevo método (Rohlf y Marcus, 1993).

La comparación de caracteres anatómicos o de referencia (hitos) entre organismos ha sido un elemento central de la biología comparada durante siglos (Adams et al. 2004). Históricamente, la clasificación taxonómica y la comprensión de la diversidad biológica han basado sus fundamentos estructuralmente en descripciones morfológicas (Adams et al. 2004). A principios del siglo XX, la biología comparada entró en una transición entre el campo descriptivo y la ciencia cuantitativa, en la que, el análisis morfológico tuvo una similar revolución cuantitativa (Bookstein, 1998).

Sobre la base de esta revolución matemática cuantitativa, el estudio de la morfología ha tenido un importante énfasis gracias al desarrollo estadístico del análisis de la "forma"; esto hizo posible la combinación de métodos estadísticos multivariados y nuevas maneras de visualizar una estructura

(Adams y Funk, 1997). Esta "Síntesis Morfométrica", conocida actualmente como Morfometría Geométrica, permite un máximo aprovechamiento de la información geométrica que posee una estructura (Rohlf y Marcus, 1993). Estas herramientas permiten el estudio de la forma integrando el tamaño de los organismos, proporcionando análisis robustos y herramientas gráficas para la cuantificación y visualización de la variación morfológica intra e interespecífica (Adams et al. 2013).

La MG permite el estudio de la forma, definida como las propiedades geométricas restantes tras eliminar los efectos de la escala, la rotación y la traslación de un objeto (Rohlf y Slice, 1990). Una técnica dentro de estos métodos de evaluación de la forma, es la búsqueda de componentes no-uniformes del cambio de la forma (Thin-plate-spline), la cual representaría todos los movimientos de los puntos anatómicos, es decir, las variaciones locales y no lineales, indicando por tanto los cambios producidos en sectores puntuales de la forma (Adams et al. 2004).

La morfometría tradicional (lineal) se basa en distancias, índices o ángulos (Tornese y Nabar, 2013). Estos métodos rara vez preservan las relaciones geométricas del objeto de estudio durante el análisis, lo que dificulta enormemente la visualización de los cambios morfológicos. Como consecuencia de lo anterior, la generación de gráficos o visualizaciones de los resultados usualmente se hace a través de tablas poco intuitivas. Aunque las distancias son inherentemente independientes de la posición y orientación del objeto del cual son obtenidas, no permiten ninguna remoción de los efectos de la escala/tamaño matemáticamente comparable a la obtenida en MG mediante el análisis de Procustes (Slice, 2005). Así la MG es una herramienta poderosa al estudio de función y evolución morfológica (Zelditch et al. 2012) y puede proveer únicas y novedosas perspectivas cuando es aplicado a Monogenea.

Los Monogeneos (Platyhelminthes) son principalmente ectoparásitos de peces marinos y agua dulce (Whittington, 2005) y mayormente restringidos a la piel y las branquias. Estos parásitos proveen una plataforma apropiada para revelar novedosas perspectivas en ecología y evolución, debido a su ciclo de vida directo y al alto nivel de preferencia por el hospedador (Pariselle et al.

2011). Los monogeneos adultos tienen sus órganos de fijación en la parte posterior de su cuerpo bien desarrollados, lo que les permite resistir el desprendimiento del sistema branquial (Šimková et al. 2001). La morfología de estos órganos de fijación, junto a la del órgano copulador son taxonómicamente las más importantes y con frecuencia son usadas para distinguir entre especies (Vignon et al. 2011). La morfología de los ganchos en la subclase Monopisthocotylea ha sido estudiada como modelos para investigar procesos que llevan a la especialización (Šimková et al. 2002; Vanhove y Huyse, 2015), para elucidar la asociación parásito-hospedador en el contexto de ecología evolutiva (Mendlová y Šimková, 2014) y para explorar la correlación entre la variación del fenotipo en los órganos de fijación y factores tales como la filogenia, especificidad hospedadora y localización geográfica (Vignon et al. 2011; Khang et al. 2016).

Entre los monogeneos, *Ligophorus* Euzet and Suriano, 1977 incluye parásitos restringidos a peces mugílidos (Sarabeev et al. 2013). Éste género es rico en especies (unas 60) (Rodríguez-González et al. 2015b) y son morfológicamente diversos y con filogenias bien resueltas (Blasco-Costa et al. 2012; Khang et al. 2016). *Ligophorus* spp. y sus hospedadores mugílidos definen un excelente escenario para las asociaciones parásito-hospedador. A nivel de especie, el patrón sobresaliente es que cada especie de *Ligophorus* predomina en una sola especie de hospedador y que a menudo coexista con una o más especies congéneres (Blasco-Costa et al. 2012). En el Mar Mediterráneo y Mar Negro existen 16 especies de *Ligophorus* conocidas y que han sido registradas en 6 especies de mugílidos (Blasco-Costa et al. 2012).

La evidencia actual sugiere que éste sistema parásito-hospedador se caracteriza por un alto grado de intercambio de parásitos de mugílidos en zonas como el Mediterráneo, Mar Rojo y Mar Negro, donde estos peces son diversos y simpátricos (Blasco-Costa, 2009). Los mugílidos albergan una diversidad de fauna en general de parásitos, que incluye representantes de los principales grupos parásitos como protozoos, helmintos y crustáceos. Entre estos, esta Tesis Doctoral abarca

todas las especies de mugílidos como hospedadores de *Ligophorus* spp. en las zonas geográficas antes mencionadas.

La variabilidad fenotípica en las estructuras del haptor en *Ligophorus* ofrece una oportunidad para investigar la variación morfológica a escala evolutiva. Dicha variación han sido estudiadas bajo dos enfoques: morfometría tradicional y MG. Sin embargo la variación morfométrica en estructuras esclerotizadas en monogeneos se ha estudiado desde el punto de vista de la sistemática (Shinn et al. 2001) y ecología evolutiva (Mendlová y Šimková, 2014). Las partes duras, como los "ganchos", son un modelo ideal para el análisis de MG, ya que no se deforman fácilmente por la compresión cuando se montan en preparaciones (Lim y Gibson, 2009).

Hasta la fecha existen pocos estudios en los que se haya aplicado éste enfoque en la variación de forma en los ganchos de los monogeneos (Vignon y Sasal, 2010; Vignon, 2011; Vignon et al. 2011; Llopis-Belenguer et al. 2015; Khang et al. 2016). La escasez de estudios en MG, sin embargo, contrasta con la importancia de este enfoque en la inovación de la variación de la forma inter e intraespecífica en los anclajes que puede ser muy valiosa para la delimitación de especies, así como en la evaluación de hipótesis de modularidad e integración morfológica entre las partes de los ganchos.

El análisis de patrones de congruencia entre filogenias de mugílidos y la morfometría de los ganchos de *Ligophorus* abre la posibilidad de determinar la importancia relativa de fenómenos de espciación alopátrica frente a los de especiación simpátrica en la formación de la diversidad de este género (Vanhove y Huyse, 2015).

Esta Tesis Doctoral está dedicada al estudio de la variabilidad fenotípica en forma y tamaño de las estructuras del haptor en 14 especies de *Ligophorus* para aportar nuevas perspectivas sobre la evolución en monogenea en la morfología de las estructuras de anclaje a sus hospedadores y de los procesos evolutivos de las asociaciones parásito-hospedador. Así como añadir el registro de una

nueva especie del género *Ligophorus* en América (región poco estudiada), y así contribuir al conocimiento de la diversidad sobre la biogeografía de este género.

Por tanto éste estudio pretende responder las siguientes preguntas:

- ¿Cuáles son los patrones de variación en forma y tamaño de los ganchos en relación al sitio de fijación?
- ¿Existe suficiente evidencia morfológica para describir una nueva especie de *Ligophorus* en América?
- ¿Qué papel desempeñan las raíces y puntas de los ganchos en las especies de *Ligophorus* respecto a su morfología funcional?
- ¿Cómo es la evolución de la morfología del gancho en las especies de *Ligophorus*? ¿Son caracteres independientes o modulares?
- ¿Existe señal filogenética en la forma y tamaño en las especies de *Ligophorus*?

Justificación y objetivos

El presente trabajo abarca dos enfoques: morfometría tradicional y morfométrica geométrica. En primer lugar contribuyendo al conocimiento de la diversidad en *Ligophorus*, describiendo una nueva especie para América, y en segundo lugar la contribución de la morfometría geométrica en determinar hasta que punto la variabilidad morfológica observada es explicada por patrones evolutivos o por homoplasia. Asímismo, los resultados de este estudio revelan patrones que permiten realizar inferencias sobre morfología funcional de las estructuras de fijación.

Los objetivos específicos que se abordan en este estudio son:

1) Analizar la variabilidad en forma y tamaño de los ganchos ventrales y dorsales en *Ligophorus cephali* en *Mugil cephalus*, por medio de morfometría geométrica y estadística multivariada, y

posteriormente evaluar la integración morfológica entre ganchos, y entre raíces y puntas de éstos.

2) Describir una nueva especie de *Ligophorus* en *M. cephalus* en la Península de Yucatán México y actualizar los registros zoogeográficos a luz de la actual evidencia de un complejo de especies crípticas.
3) Determinar si la variación en la forma de los ganchos en las 14 especies de *Ligophorus* es modular e integrada, evaluada por 4 hipótesis de modularidad en ambos niveles: morfológico y evolutivo.
4) Evaluar si existe señal filogenética en la forma de los ganchos ventrales y dorsales en 14 especies de *Ligophorus* en 6 especies de mugílidos del Mar Mediterráneo, Mar Negro y Azov, para demostrar si la similitud en los ganchos es debido a la convergencia o a la historia evolutiva.

Material y Métodos Generales

En este resumen se describen los materiales y métodos generales de forma condensada, así como los análisis geométricos y filogenéticos. Los análisis estadísticos se explican en el apartado correspondiente de cada capítulo.

Área de estudio y muestreo de los hospedadores

Se tomaron muestras de mugílidos en seis localidades. En la costa Mediterránea española, frente al Delta del Ebro (40°30'-40°50'N, 0°30'-1°10'E), en la bahía de Santa Pola (38°00'-38°20'N, 0°10'-0°40'W) y la Albufera (39°20'0"N-0°21'0"W). Adicionalmente y para completar el estudio se obtuvieron muestras del Estrecho de Kerch, Mar de Azov (45°16'20.8"N-36°31'40.6"E) y delta del río Artemovka, Mar de Japón (43°18'30.3"N-132°17'4.8"E). Para conocer la biodiversidad en América, se colectaron peces de la Laguna de Celestún, Yucatán México (20°51′33″N-90°24′00″O).

Los peces colectados en ésta tesis fueron adquiridos de los pescadores en mercados locales durante la primavera (2004), otoño (2005), primavera-otoño (2011) y la primavera (2014). Seis especies de mugílidos fueron examinados: *Mugil cephalus*; *Liza haematocheila*; *Lz. aurata*; *Lz. ramada*; *Lz. saliens* y *Chelon labrosus*. Los peces fueron examinados el mismo día de captura o después de estar congelados. El número de peces usados en este estudio fueron 121. Los peces del mar Mediterráneo fueron sacrificados e inmediatamente fueron puestos en neveras con hielo para ser transportados al laboratorio de la Unidad de Zoología Marina. Posteriormente, se registró la longitud total y el peso de cada especie de pez.

Procesamiento de los monogeneos parásitos y análisis morfológico

Para identificar los parásitos a nivel de género se basó en la observación del complejo de forma y tamaño de los ganchos ventrales y dorsales, barras desiguales con presencia de protuberancias anteriores, presencia de siete pares de microganchos, así como la morfología del órgano copulador (Sarabeev et al. 2013).

Las branquias de cada hospedador de mugílidos fueron extraídas y la superficie de cada branquia fue individualmente examinada y separada por el lado derecho e izquierdo para la observación de monogeneos. Las branquias fueron examinadas en fresco para colectar en vivo el material para los posteriores análisis morfológicos, morfométricos y genéticos. Las especies de *Ligophorus* fueron contadas e identificadas de acuerdo a Sarabeev et al. (2013). Las branquias infectadas de monogeneos fueron puestas en un bote de plástico con 4% de formalina por 3-4 horas para mantener los monogeneos fijados a su específico sitio de selección en las branquias (Rodríguez-González et al. 2015a).

Para los análisis morfométricos y geométricos, se utilizó una técnica enzimática para obtener solo las estructuras esclerotizadas de cada especie de *Ligophorus* y facilitar, de esta manera, su observación al microscopio. Se usó una preparación de 300 µl de TE9 buffer (500 mM Tris-HCl, pH

9) y 100-200 µl de proteinasa K (10 mg/ml) (Paladini et al. 2011). Las muestras se identificaron de acuerdo a los caracteres morfológicos de acuerdo con Rubtsova et al. (2006), Dmitrieva et al. (2009) and Sarabeev et al. (2013).

Después de la digestión, los especímenes fueron montados en glicero-gelatina (Sarabeev et al. 2013), y otros fueron preservados en alcohol 70%, y posteriormente teñidos con acetocarmín, deshidratados mediante una cadena de alcoholes (70-100%), aclarados en ftalato de dimetilo y montados en bálsamo de Canadá para la observación de su anatomía interna (ver más detalles en el capítulo 5).

Los ejemplares de *Ligophorus* fueron observados bajo un con un microscopio óptico con ayuda de contrastes diferenciales de interferencia DIC. Las medidas se dan en micrómetros como media ± desviación estándar, seguido por los rangos en paréntesis.

Morfometría Geométrica

En esta tesis solo los ganchos (ventrales y dorsales) en buena condición de especímenes adultos de ambos lados fueron considerados. Específicamente un gancho de cada par (izquierdo o derecho) de cada especímen. Los ganchos fueron dibujados usando un tubo de dibujo a 100× aumentos (aceite de inmersión) en un microscopio Nikon Optiphot-2 con contraste de interferencia. Asimismo se tomaron fotografías en algunos casos con una cámara digital Leica DC150 en contraste de interferencia.

Los análisis morfométricos de este estudio se basaron en dos-dimensiones (Zelditch et al. 2012). Esta técnica permite separar los dos componentes de variación de la forma: tamaño y forma (geométrica) y visualizar los resultados como cambios de la forma en regiones específicas en estructuras biológicas bajo examen. Las imágenes brutas fueron compiladas y escaladas con tpsUtil version 1.52 (Rohlf, 2012).

Se escogieron 8 puntos anatómicos homólogos en los ganchos para todas las especies de *Ligophorus*. Para seleccionarlos, se tuvieron las siguientes consideraciones: 1) la configuración de un punto anatómico tiene que ser homólogo (reconocible en todos los especímenes). 2) las configuraciones de puntos anatómicos deben ofrecer un adecuado resumen de la morfología de los ganchos. 3) los puntos anatómicos tendrán que ser consistentemente replicables con un alto grado de exactitud. 4) los puntos anatómicos tienen que ser coplanares.

Posteriormente se extrajeron las coordenadas cartesianas de cada punto anatómico por medio del programa tpsDig versión 2.17 (Rohlf, 2013).

El análisis más importante de la morfometría geométrica es el análisis de superimposición de Procrustes (Klingenberg, 2010), donde la información de la forma es extraída y los componentes de variación del tamaño; posición y orientación son eliminados (Goodall, 1991; Rohlf, 1999). Los componentes superfluos de variación se eliminaron reescalando las configuraciones a un tamaño estándar, ajustando a una posición y rotando a una orientación estándar (Bookstein, 1996) para cada especie de *Ligophorus* para ganchos ventrales y dorsales. El tamaño del centroide se cuantificó como medida del tamaño y se calculó como la raíz cuadrada de la suma de las distancias de los puntos anatómicos al centro de gravedad de una configuración (Zelditch et al. 2012).

La variación de la forma se presentó en un espacio de la forma. Dicho espacio representa todas las formas posibles dado un número de puntos anatómicos, tal que las distancias entre los puntos representen similitudes entre las formas correspondientes (Klingenberg, 2010). Los cambios de la forma se visualizaron en placas delgadas de deformación y de contornos (Klingenberg, 2013).

Todos los análisis morfométricos, a menos que se indique lo contrario, fueron llevados a cabo con el programa MorphoJ versión 1.06d (Klingenberg, 2011).

Alometría y corrección del tamaño

Para evaluar la alometría (la independencia de la forma con respecto al tamaño) (Klingenberg, 2016), se utilizó una regresión multivariada de las coordenadas de Procrustes (variable de forma) contra el logaritmo transformado del tamaño del centroide (variable de tamaño) para eliminar los efectos del tamaño sobre la forma. La regresión se ajusta una línea recta a los puntos de datos que representan la forma prevista para cada valor de tamaño. Las desviaciones de un punto de esa línea –los residuales– representan la variación que no es explicada por el tamaño. Por lo tanto, se implementó una regresión multivariada usando los residuales de la regresión de la forma de los ganchos con el tamaño para corregir su efecto (Klingenberg y Marugán-Lobón, 2013).

Análisis molecular

Para evaluar la plasticidad fenotípica de *Ligophorus cephali*, primero se realizó el análisis molecular de ITS1 para confirmar que todos lo individuos fueran de la misma especie (ver capítulo 4). Para realizar dicho procedimiento, se secuenció y comparó el ITS1 del rDNA.

Los especímenes utilizados en análisis moleculares se fijaron en alcohol 100% y se guardaron a -20°C para ser transferidos posteriormente a un tampón en 200 µl de TE9 buffer (500 mM Tris-HCl, 200 mM EDTA, 10 mM NaCl, pH 9) (Wu et al. 2007). El ADN fue extraído utilizando un individuo a la vez mediante el kit comercial Qiagen DNeasy® Blood & Tissue siguiendo las instrucciones del fabricante. Las secuencias de ITS1 fueron amplificadas usando los primers Lig18endF and Lig5.8R (Blasco-Costa et al. 2012). La amplificación de las secuencias se realizó mediante la reacción en cadena de la polimerasa (PCR) en reacciones de 20 µl conteniendo 2 µl de ADN extraído. Para la PCR de amplificación se utilizó 2x MyFi Mix y 5pmol/µl of de cada primer.

Los perfiles del termociclador aplicados fueron: desnaturalización del ADN a 95° durante 3 min, 35 ciclos de amplificación con 40 de desnaturalización a 94 °C, alineando los primers a 56 °C y 45 s a 72 °C para la extensión del primer, y un paso de extensión final de 4 min a 72 °C.

Las amplificaciones de PCR fueron purificadas y los primers del PCR fueron usados para secuenciar. Las secuencias se realizaron con un kit comercial de secuenciador automatizado ABI 3730XL. Las secuencias contiguas fueron ensambladas y editadas con los programas VectorNTI avanzado 10 (Lu y Moriyoma, 2004). Posteriormente, fueron verificados utilizando la herramienta BLAST (Benson et al. 2005). Las nuevas secuencias generadas fueron alineadas para su comparación utilizando MUSCLE (Edgar, 2004) (ver más detalles en el capítulo 4).

Análisis Filogenético

Para obtener la filogenia de las especies de *Ligophorus* del mar Mediterráneo, se utilizaron las secuencias 28S rDNA y ITS1 dispnibles en GenBank (Blasco-Costa et al. 2012) (ver capítulo 6). Las secuencias de cada gen fueron alineadas (Tamura et al. 2013). Previamente al análisis, se estimó el mejor modelo de substitución nucleotídica utilizando el programa jModelTest versión 2.1.6 (Darriba et al. 2012). El modelo seleccionado se especifica en el capítulo 6. Las secuencias alineadas de los dos genes fueron concatenados. Para el análisis de máxima verosimilitud (*Maximum-likelihood*) un árbol de partida fue construido basado en neighbor joining. El soporte de las ramas fue estimado por el análisis de bootstrap con 1000 réplicas. El análisis de inferencia bayesiana (IB) fue utilizado con cuatro cadenas de Márkov de Monte Carlo para 10^6 generaciones con una frecuencia de muestreo de 1000 y utilizando el primer quartil de los árboles almacenados como set de entrenamiento (*burn-in*). El árbol consenso fue construido omitiendo los árboles de este primer conjunto de árboles. El soporte nodal se estimó como probabilidades posteriores (Huelsenbeck et al. 2001). Véase el capítulo 6 para más detalles.

Debido a que los árboles obtenidos, bayesiano y máxima verosimilitud, fueron muy similares, sólo se usó el primero para proyectarlo sobre la forma y tamaño en los morfoespacios usando un análisis de componentes principales. Esto fue hecho sopreponiendo los valores de los componentes principales (CP) de las especies, a la filogenia usando squared-change parsimony y calculando los

valores de CP en los nodos internos (Maddison, 1991; Klingenberg y Gidaszewski, 2010) (ver detalles en el capítulo 7).

Plasticidad fenotípica en las estructuras del haptor de *Ligophorus cephali* (Monogenea: Dactylogyridae) en *Mugil cephalus*: un enfoque geométrico morfométrico

El estudio morfológico y plasticidad fenotípica en las estructuras esclerotizadas del haptor en los monogeneos aplicando técnicas de morfometría geométrica ha sido un campo poco explorado (Vignon y Sasal, 2010). En este estudio se documentó por primera vez la integración morfológica entre los ganchos ventrales y dorsales, y entre las raíces y puntas de los ganchos en *L. cephali*, para dar a conocer información detallada de las variaciones de la forma entre esos ganchos, así como modelar la forma morfológica y tamaño en función de las variables del hospedador, tales como: arco branquial, área branquial, sección branquial y hospedador individual.

Las deformaciones determinaron y dividieron la variación de la forma en componentes uniformes (variación global) y no-uniformes (variación local) (Zelditch et al. 2012). A nivel global, los patrones de variación de la forma fueron similares en los ganchos ventrales y dorsales, definiendo un gradiente que va, desde formas estrechas y alargadas, a anchas y ganchos cortos. La variación de la forma fue mayor en los ganchos dorsales, lo cual está en línea con los altos valores de variación residual asociada con los ganchos dorsales en los modelos de la forma (ver capítulo 4). Además en los modelos con el tamaño, la variación residual de los ganchos dorsales fue mucho mayor que en los ganchos ventrales. Se mostró que los efectos de sección branquial × hospedador individual fueron un importante determinante en la forma de los ventrales, pero no en los dorsales. Esta evidencia reflejó que las diferencias entre los ganchos ventrales y dorsales en los factores que determinan la forma y tamaño podrían ser fruto de papeles funcionales diferentes en la fijación a la branquia. La

morfología del gancho y barra dorsal es más movible que la ventral, lo que apoya también la posibilidad de diferencias funcionales.

La baja variación residual en los ganchos ventrales sugiere un alto control en su forma y tamaño, debido a que posiblemente estos ganchos son los más importantes en la fijación y su tamaño y forma podrían ajustarse más a las características del microambiente provisto por el hospedador (Šimková et al. 2001, Sarabeev et al. 2013).

Se observó alta integración morfológica en la forma entre los ganchos ventrales y dorsales, sugiriendo fuerte coordinación (Vignon et al. 2011). Esta coordinación sugirió que esta covariación morfológica desempeña un papel substancial en determinar el potencial evolutivo de los caracteres dentro de las poblaciones. Asimismo se observó integración dentro de la estructura del gancho (raíces y puntas).

Una considerable parte de la variación en los modelos de forma y tamaño fue atribuible a factores aleatorios no explicados por las variables consideradas. Existió, por tanto, un gran componente no predecible en los modelos imputables a la combinación de medidas de error, variación genética, cambios ontogenéticos y respuestas plásticas a los factores ambientales. Los análisis moleculares revelaron que la secuencias ITS1 fueron idénticas a las registradas previamente por Blasco-Costa et al. (2012). Esta evidencia no descarta completamente algún nivel de variación genética en nuestra muestra, por lo tanto es posible que no toda la variación fenotípica revelada sea ambientalmente inducida.

Adicionalmente el arco branquial del hospedador fue un importante determinante en la forma y tamaño del gancho en los ganchos dorsales. La evidencia presentada aquí puntualiza que la plasticidad fenotípica en la morfología del gancho puede atribuir a *Ligophorus* la habilidad de colonizar un nuevo hospedador.

Nueva especie de *Ligophorus* (Monogenea: Dactylogyridae) en las branquias de *Mugil cephalus* (Teleostei: Mugilidae) de México

Las especies de *Ligophorus* son restringidas a mugílidos en el mundo (Sarabeev et al. 2013). A la fecha sólo un registro de *Ligophorus* fue reportado en el Golfo de México (Hargis, 1955). En este estudio colectamos especímenes de *Ligophorus* en la costa de Yucatán y su estudio morfológico sugiere que representan a nueva especie. *Ligophorus yucatanensis*, que es añadida a las 60 especies reconocidas en *Ligophorus* en el mundo. La nueva especie puede ser diferenciada de todas las especies de *Ligophorus* por la morfología de la pieza accesoria del órgano copulador. Esta presenta el lóbulo principal cilíndrico, en forma de túnel expandido distalmente, ligeramente inclinado con una estructura membranosa abierta a nivel de la bifurcación de la pieza accesoria, formando una pared gruesa en el bulbo que termina en un borde redondo.

L. yucatanensis presenta el lóbulo secundario espatulado del órgano copulador, estrecho y más corto que el lóbulo principal. Además, la nueva especie puede ser distinguida de otras especies por la morfología de la barra ventral del haptor y por la posición distal del ducto vaginal. Otra característica es que los ganchos ventrales son más cortos que en especies de *Ligophorus* reportadas en el Golfo de México y en el Caribe.

La nueva especie se asemeja más a las especies del mar Mediterráneo y costa del noroeste Pacífico más que las especies registradas en Sudamérica y Norteamérica. La prevalencia de *L. yucatanensis* fue de 77% lo que está en el rango reportado para las especies del Caribe (48-100%) (Sarabeev et al. 2005).

Dentro del registro zoogeográfico reportado por El Hafidi et al. (2013) en *Ligophorus* spp. del complejo de especies de *M. cephalus*, se reconocieron 14 entidades geográficas del hospedador; la nueva especie debe adscribirse a la entidad 4 (ver capítulo 5) donde solo *L. mugilinus* había sido

registrado. Debido a la condición y al bajo número de especímenes disponibles no se pudo realizar el estudio molecular con objeto de determinar la posición filogenética de la nueva especie.

Modularidad evolutiva e integración morfológica de las estructuras del haptor de *Ligophorus* spp. (Monogenea: Dactylogyridae)

En este estudio, se utilizó morfometría geométrica en un contexto comparativo lo que permitió estudiar los mecanismos de modularidad e integración por medio de las configuraciones de los puntos anatómicos en 14 especies de *Ligophorus*. Los resultados mostraron que existió una fuerte integración evolutiva indicando que los ganchos ventrales y dorsales evolucionan como una estructura simple e integrada para la fijación a las branquias del hospedador.

El efecto del tamaño (alometría) fue un factor que contribuyó a la evaluación de módulos, éste solo explico una pequeña porción de la variación de la forma de los ganchos y su influencia estadística no fue significativa cuando la filogenia del parásito se tomó en cuenta.

La variación de los ganchos en *Ligophorus* spp. se concentró en los compartimentos modulares de fijación: raíces y puntas para los ganchos ventrales y dorsales. La forma de los pares de ganchos dorsal-ventrales mostró un nivel de covariación significativo en la especies de *Ligophorus*, indicando dos módulos independientes. La ocurrencia de módulos en raíces y puntas en los ganchos ventrales y dorsales no fue corroborada cuando la filogenia del parásito fue incluida, lo cual pudiera ser indicativo de evolución convergente (Khang et al. 2016). Las respuestas adaptivas del microhábitat en branquias pudo haber facilitado la formación de estos módulos. Así, la homoplasia podría explicar las restricciones de la forma impuestos por la filogenia (Sarabeev y Desdevises, 2014).

El estudio demostró, asimismo, una bipartición de variación morfológica en ganchos ventrales y dorsales formando dos módulos, lo que sugiere que cada tipo de gancho tiene diferente papel funcional de fijación a las branquias (Vignon et al. 2011; Khang et al. 2016). Así mismo, la covariación de la forma representó dos módulos evolutivos independientes. En este escenario

evolutivo, los módulos separando las partes medial y lateral de los ganchos se mantuvieron en los ganchos ventrales cuando se aplicó la técnica de contrastes independientes filogenéticos (PIC). Por lo que la filogenia demostró ser el mayor determinante de la variación de la forma en estos ganchos. Por otro lado, la inexistencia de módulos con las otras hipótesis probadas podrían ser explicados por divergencia, debido a la adaptabilidad de las especies de *Ligophorus* a diferentes microhábitats en las branquias (Khang et al. 2016).

La existencia de los módulos funcionales indentificados en este estudio concuerda con la arquitectura muscular de los órganos del haptor de *Ligophorus* spp. (Petrov et al. 2015).

Morfología evolutiva en forma y tamaño en 14 especies de *Ligophorus* (Monogenea: Dactylogyridae)

Los patrones de cambios morfológicos en los ganchos del haptor fueron interpretados para reconstruir las dinámicas en los procesos evolutivos y fueron visualizados como trayectorias de los antecesores por el morfoespacio (Klingenberg, 2010).

Dado que existe una gran variabilidad en la forma de los ganchos en *Ligophorus* no fue sorprendente que ellos cubrieran un rango de formas en el espacio tangente (Ver capítulo 7). Los análisis llevados a cabo en este estudio demuestran una fuerte señal filogenética determinando la forma y en menor grado el tamaño en los ganchos del haptor. Evaluamos la hipótesis de que el hospedador puede tener una influencia sobre la genética o morfología en monogeneos (Desdevises et al. 2002) y que la morfología del haptor refleja adaptaciones al sistema de fijación al hospedador (Šimková et al. 2002) en un marco teórico morfométrico, comparando la posición en un filomorfoespacio de especies distantemente relacionadas que co-ocurren en diferentes especies de hospedador.

L. confusus y *L. imitans* que parasitan a *Lz. ramada* representan diferentes clados y sus ganchos aparecieron separados en forma y tamaño en el morfoespacio. Igualmente, *L. szidati* y *L. vanbenedenii*

que co-occurren en *Lz. aurata* aparecen en clados diferentes y difirieron marcadamente en forma y tamaño para los ganchos dorsales. Por tanto, no se encontró clara evidencia de homoplasia determinada por la especie hospedadora. En varios casos, miembros de un mismo clado que ocurren en la misma especie de hospedador mostraron forma similares de ganchos (*L. cephali* – *L. mediterraneus* en *M. cephalus*, *L. llewellyni* y *L. pilengas* en *Lz. haematocheila*) o formas similares (*L. acuminatus* y *L. minimus* en *Lz. saliens*). Estos clados probablemente resultaron de duplicaciones en el hospedador (Blasco-Costa et al. 2012) y sus similitudes morfológicas señalan la ocurrencia de restricciones filogenéticas en la forma del gancho (Mendlová y Šimková, 2014).

Se observó que especies hermanas que ocurrieron en diferentes hospedadores mostraron similitudes en forma y a veces en tamaño (*L. imitans* y *L. szidati* con sus respectivas especies hermanas *L. heteronchus* y *L. confusus*). La posición filogenética de *L. imitans* mostró afinidades con las especies encontradas en *Lz. saliens*, lo que sugiere que la ocurrencia en *Lz. ramada* representa un evento de captura hospedador. La adaptación a nuevos hospedadores no impuso cambios drásticos en la morfología de los ganchos, reafirmando la idea de que la filogenia es el mayor determinante de la morfología de los ganchos del haptor en *Ligophorus*.

Este estudio permitió, además, identificar trayectorias morfológicas de evolución. En el clado de *L. heteronchus* – *L. cephalus* las especies basales (*L. heteronchus* – *L. macrocolpos*) están asociadas a *Lz. saliens*, mostrando ganchos angostos con el mango del gancho largo. Esta forma representa probablemente el estado ancestral relativo a la morfología derivada de los ganchos en el clado *L. llewellyni* – *L. cephali*, los cuales incluyeron formas en *Lz. haematocheila* y *M. cephalus* caracterizados por raíces largas. Las posiciones en el morfoespacio apoyan la idea de que la ocurrencia en *Ligophorus* en *Mugil* se explica por eventos de captura de hospedador de *Liza* – *Chelon* que ocurrieron fuera del Mediterráneo.

Una combinación de especies de hospedador y microhábitat podrían haber contribuído para explicar la alta diversidad de las formas de los ganchos dorsales.

CONCLUSIONES

El presente estudio demostró el potencial de la morfometría geométrica para inferir nuevas visiones sobre la morfología funcional del anclaje en las especies de *Ligophorus* y de los procesos evolutivos de coevolución parásito-hospedador. Como síntesis del estudio, éstas son las principales conclusiones:

1. En este estudio se encontró que el patrón de variación de la forma fue similar en ganchos ventrales y dorsales. La variación de la forma localizada fue mucho mayor en los ganchos dorsales, lo cual coincidió con la alta variación residual en los modelos de la forma. Los efectos aleatorios (sección de la branquia × hospedador individual) fueron un importante determinante en los ganchos ventrales, pero no en los dorsales. Los modelos de tamaño en los ganchos fueron diferentes. La evidencia reflejó diferenciales funcionales en el anclaje a las branquias. Las diferencias en morfología del gancho/barra dorsales más móviles y ventrales más rígida dieron soporte a ésas diferencias.
2. Se observó una alta integración morfológica en la forma entre los ganchos ventrales y dorsales, lo cual sugiere que actúan concertadamente. Por el contrario, se observó baja integración dentro de la estructura del gancho del parásito (raíces y puntas), lo que sugiere que una fracción de la variedad fenotípica observada no compromete el papel funcional de los ganchos como palancas.
3. El arco branquial fue un importante determinante de la forma y tamaño del gancho en ganchos dorsales. La variabilidad de la forma se puede asociar a los procesos hidrodinámicos junto con la posición espacial de cada branquia. La plasticidad fenotípica en *Ligophorus* spp.

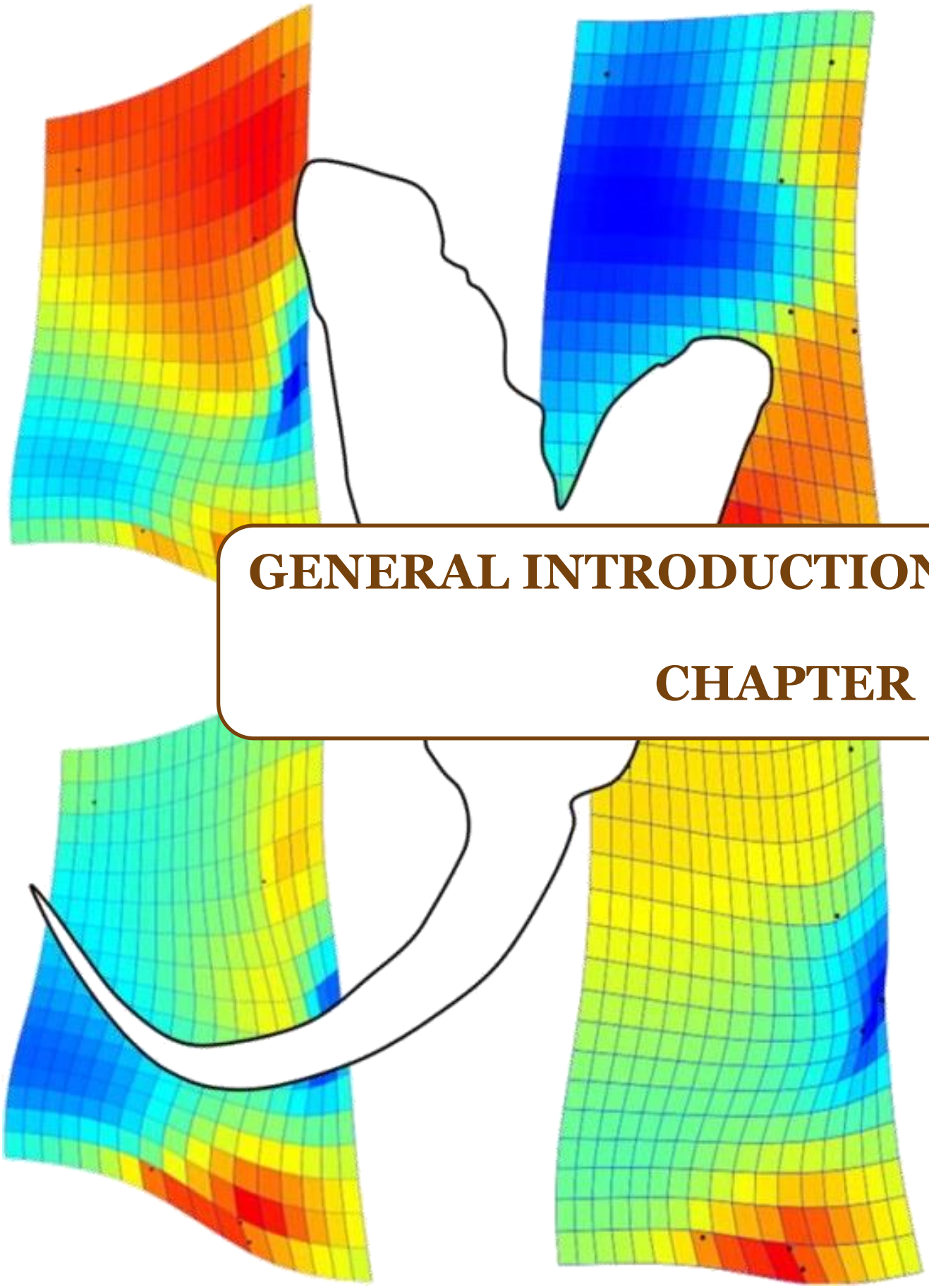
podría indicar la capacidad para colonizar a nuevos hospedadores. Por otro lado, no se encontró una correlación entre el tamaño de los ganchos y el tamaño del hospedador.

4. Se demostró que la morfometría geométrica puede ser una técnica extremadamente útil en analizar la variación intraespecífica en forma y tamaño en las estructuras del haptor en monogeneos y así dar lugar a nuevos enfoques en el estudio de la morfología funcional del anclaje y procesos evolutivos entre parásito y hospedador.
5. Para conocer la diversidad en monogeneos en otras áreas geográficas, se describió una nueva especie de monogeneos *Ligophorus yucatanensis* en las branquias de *Mugil cephalus* en la Península de Yucatán. La nueva especie se diferenció de todas las demás especies de *Ligophorus* por la morfología de la pieza accesoria del órgano copulador, por la morfología de la barra ventral y por la parte terminal del ducto vaginal. Los ganchos ventrales fueron más cortos a comparación de los reportados en el Golfo de México y Mar Caribe.
6. *L. yucatanensis* es más parecida a las especies del Mediterráneo y a las costas de Noroeste del Pacífico, que las especies registradas en Sur y Norteamérica.
7. La nueva especie fue incluída dentro de la entidad 4 (Noreste Atlántico) de acuerdo a los registros zoogeográficos de *Ligophorus* spp. del complejo de especies *M. cephalus*.
8. Se aporta evidencia por medio de Contrastes Independientes Filogenéticos (PIC) y morfometría geométrica que indica que la variación de la forma en los ganchos de *Ligophorus* estuvo concentrada en algunos módulos: raíces y puntas para los ganchos ventrales y dorsales, par de ganchos dorsal-ventral que representarían dos módulos independientes para el anclaje en las branquias. La complejidad del microhábitat (arco branquial, segmento o área) provistas por las branquias del hospedador y las respuestas adaptivas por los monogeneos pudieron haber facilitado la formación de módulos diferenciados entre raíces y puntas de los ganchos. La integración morfológica de ésos módulos fue significativa. La modularidad a nivel evolutivo no fue corroborada, lo cual parece indicar evolución

convergente, sólo en los módulos parte media y lateral del gancho se mostró congruencia significativa con PIC en los ganchos ventrales. La filogenia fue el mayor determinante de la variación en la forma de los ganchos. Por otro lado, la presencia de modularidad a nivel evolutivo se explicó por divergencia debido al incremento en adaptabilidad a las diferentes microhábitats en las branquias.

9. La disposición muscular en las especies de *Ligophorus* es congruente con la formación de los módulos funcionales para el anclaje a la branquia y para integración en los ganchos ventrales y dorsales.
10. La alometría en los ganchos ventrales y dorsales en las especies de *Ligophorus* fue un factor que contribuyó en la evaluación de la modularidad, explicando sólo una pequeña porción de la variación de la forma. Este factor no fue significativo cuando la filogenia fue tomada en cuenta.
11. Dada la variedad de formas en los ganchos de *Ligophorus* spp., estos cubrieron un rango substancial en el rango de formas en el filomorfoespacio. La presencia de señal filogenética indica que los procesos evolutivos juegan un papel mayor en determinar la forma y en menor grado el tamaño en los ganchos del haptor.
12. Se evaluó la hipótesis de que la morfología del haptor refleja adaptaciones al anclaje del hospedador, comparando la posición en el filomorfoespacio de especies relacionadas distantemente que co-ocurren en una especie de hospedador. Dado que los ganchos de especies filogenéticamente distantes que coocurren en la misma especie hospedadora eran claramente diferentes, se concluyó que no existe evidencia clara a favor de homoplasia determinada por la especie de hospedador.
13. La evaluación de la señal filogenética en los caracteres morfológicos usando morfometría geométrica sirvió para estimar los pesos relativos de convergencia e historia evolutiva determinando la morfología de los ganchos en *Ligophorus* spp.

14. Los resultados de este estudio sugieren que la historia evolutiva juega un papel preponderante en determinar la forma y, en menor grado, el tamaño en los ganchos de *Ligophorus* spp.



GENERAL INTRODUCTION

CHAPTER 1

"The study of form may be descriptive merely, or it may become analytical. We begin by describing the shape of an object in the simple words of common speech: we end by defining it in the precise language of mathematics; and the one method tends to follow the other in strict scientific order and historical continuity"

D' Arcy Thompson (1915)

1.1. Evolutionary morphology

The morphology is the science of form, the study of how and why organisms look the way they do, of why certain parts of an organism possess certain bases. Post-Darwinian evolutionary morphology is considered in certain aspects a functional morphology. Morphology comprises various areas of research such as descriptive morphology, in which organism and their parts are described, functional morphology, which study the relationship between the structure and function of morphological features and comparative morphology, that is the analysis of the patterns of the locus of structures within the organism, and forms the basis of taxonomical categorization. (Richter and Wirkner, 2014). A central concept of Darwinian evolutionary theory and of the modern synthesis is that specific traits are the results of adaptation, and that adaptation is forced by natural selection (Losos, 2011). This implies that evolutionary phenotypic transformations can be explained on one level, in terms of the selective advantage of one evolutionary unit over another of the same transformation given a certain environment (Richter and Wirkner, 2014). In contrast, D'Arcy Thompson (1917) in his book *On Growth and Form*, argued that evolutionary transformations in the shape of organisms can be described with mathematical expressions based on the physical laws of the forces acting upon them. D'Arcy Thompson famously illustrated evolutionary transformations by deforming grids to show that the shape of one organism could be modified to produce the shape another. His artistically constructed grid deformations were the inspiration behind the development of Geometric Morphometrics (GM) in the 1980s. However, Thompson considered not only the deformation of shape but also the structural efficiency and mechanical forces related to those deformations (Polly et al. 2016).

The study of organismal shape has a long history in biology; hence, trends in shape evolution are well described. The evolutionary study of biological shape faces at least three challenges. First, shape is an inherently complex (a multivariate trait) and so shape quantification can be difficult. Second, the components of shape tend to covary strongly within biological groups (e.g. populations, species) and, therefore, the assumption of independence of observations upon which most mainstream statistical tests rely does not hold. Third, until recently, uncovering generalities that underlie the regulation and integration of trait growth had proven elusive, precluding the development of a general theory of shape expression and evolution (Cooke and Terhune, 2015).

For much of the 20th century, morphometric analyses were based in measures of traits that included linear distances, ratios, and angles (traditional morphometrics) (Cardini and Loy, 2013). While powerful in many ways, and still a mainstay in many fields, these methods lack the ability to characterize the entire shape of an organism and the measurements themselves are often treated as independent of one another, although they are part of a larger structure and may, therefore, covary. However, a radical shift in the way the shapes of anatomical structures were quantified emerged at the end of the century. This alternative captured the geometry of the morphological structures and retained the pure shape information. It was called **Geometric Morphometrics** and this paradigm shift has been saluted as a "revolution in morphometrics" (Corti, 1993; Adams et al. 2004).

Geometric morphometrics can be defined as the quantitative study of biological shape, its variation, and its covariation with other biotic or abiotic variables or factors (Webster and Sheets, 2010).

This discipline relies on homologous or analogous points on a given structure, rather than pairs of points or ratios. This approach affords cleanly partitioning the mathematical effects of size on shape and visualizing results as graphical transformations of the actual shape of the object. Landmarks represent mathematically, points of correspondence between specimens. The most common comparative method used is generalized Procrustes superimposition in which each set of

landmarks of a given specimen is rescaled, aligned at geometric centers (centroids) with other sets of other specimens (centroids) and rotated until the sum of squared distances between the corresponding landmarks is minimized (Polly et al. 2016). The removal of size, orientation, and translation reduce the degrees of freedom of the Procrustes aligned coordinates (loss of 4 degrees of freedom for 2D landmarks). The reduced dimensionality constrains variation such that shapes are distributed in a non-Euclidean mathematical space with the form of a hemisphere. Because of the non-Euclidean geometry of shape space, Procrustes coordinates are usually projected to a Euclidean tangent space, although in practice this is often unnecessary for biological shapes because developmental and functional integration typically constrains shape variation sufficiently and the nonEuclidean curvatures of shape space are irrelevant (Slice, 2001; Zelditch et al. 2012; Polly et al. 2016).

Geometric morphometrics quantifies differences in morphological shape, including static differences between individuals, sexes, or species, as well as transformational differences between ontogenetic stages, between stratigraphic units, or along branches of a phylogenetic tree (Zelditch et al. 2012). Combined with multivariate statistics and phylogenetics, geometric morphometrics can be used to analyze the relationship between shape and a variety of evolutionary, developmental, ecological, and functional factors (Adams et al. 2013). Geometric morphometrics is a powerful important tool to study of function and evolution of morphology, and can provide unique insights when applied to Monogenea, the subject of study of the present thesis.

1.2. The Monogenea

The Monogenea is a class of flatworms (Platyhelminthes) that are primarily ectoparasites of fishes, mostly restricted to the skin and gills (Whittington, 2005). Due to their direct life-cycles and relatively high level of host preference, they have been considered as suitable parasites for revealing novel insights into host ecology and evolution (Pariselle et al. 2011). Monogeneans are

morphologically diverse and represent a speciose taxon (Mendlová and Šimková, 2014). They are hermaphrodite and the life cycle is direct (monoxenous) involving a free-swimming ciliated larva, called oncomiracidium, responsible for infecting new hosts (Kearn, 2014). Adult monogeneans possess well-developed attachment organs located in the posterior part of their body, forming the haptor that helps to resist physical dislodgement from the host surface.

Monogeneans can be divided into two major groups, the monopisthocotyleans, which have hook-like organs on their haptors to attach to their host, and the polyopisthocotyleans, which use clamp-like structures for attachment. The anchors are used to attach to the host by penetration of the epithelia and often supported by transverse bars or accessory sclerites, which provide stabilization and/or attachment. In some cases, these anchors are capable of counter-rotation, which serves to spear the secondary gill lamellae. Polyopisthocotylean are too large to fit between lamellae, have downplayed their hooks and acquired remarkable clamp-like organs, which are capable of gripping one or two secondary gill lamellae. The two opposing jaws of each clamp are supported by a framework of hard sclerites (Kearn, 2014).

As monogeneans are mostly soft bodied and hence highly plastic in body shape, their hard sclerotized structures, which include their attachment and copulatory organs, are taxonomically most important and often used to distinguish between species (Vignon et al. 2011). In addition to giving important taxonomic information, the morphology attachment organs, such as anchors, in monogeneans has been extensively studied in various ecological and evolutionary contexts, because it can influence the specificity, specialization, and reproductive isolation among conspecifics through niche segregation (Vignon et al. 2011).

Monogenea has several desirable features that make them invaluable as a model system for studying evolutionary processes that resulted in its past diversification and present diversity (Poulin, 2002). Mainly many of genera are speciose, morphologically diverse, show well-resolved phylogenies at the familial level, and samples can be easily obtained in large numbers (Khang et al.

2016). They have been used to shed light on ecological forces that shape species community and structure, to investigate processes leading to speciation and its maintenance (Šimková et al. 2002; Vanhove and Huyse, 2015), to elucidate host-parasite evolutionary ecology (Mendlová and Šimková, 2014), and to explore the extent of correlation between phenotype variation in attachment organs and factors such as phylogeny, host specificity and geographical location (Vignon et al. 2011; Khang et al. 2016).

Strict host-specificity is a common phenomenon among monogeneans (Mariniello et al. 2004) and is considered to be a result of various factors including phylogenetic, physiological and ecological aspects (Mendlová and Šimková, 2014). Therefore the morphological evolution of the haptor is usually considered as the result of adaptive processes. However, the morphological determinants of anchors are not fully understood and morphological variations may be constrained by both phylogeny and local adaptation to a host or local environment (Poisot and Desdevises, 2010). These aspects are addressed in this Doctoral Thesis.

1.2.1. *Ligophorus* (Monogenea: Dactylogyridae)

Among monogeneans, *Ligophorus* Euzet and Suriano, 1977 includes specific gill parasites of grey mullets (Mugilidae). This genus is characterized by the combination of the following features: vas deferens on the left side not encircling the intestinal caeca; one prostatic reservoir; copulatory complex comprising a copulatory organ with bilobed based and an accessory piece; a J- to U-shaped ovary, a vagina sclerotized or not; dorsal and ventral anchor/bar complex with seven pairs of hooks and bars dissimilar in shape, ventral bar with anteromedian protuberances (Sarabeev et al. 2013) (Figure 1.1).

Species discrimination within the genus relies on the morphology and size of sclerotized elements of the haptor and of the male copulatory complex (Figure 1.2) (Sarabeev et al. 2013; Marchiori et al. 2015). Examination of host-parasite associations indicates that often one given host

species harbours several species of *Ligophorus* (Sarabeev and Balbuena, 2004). In fact, Euzet and Suriano (1977) considered that species of *Ligophorus* are oioxenous given that their occurrence on atypical hosts is rare and often limited to single specimens. However, a number of studies report species of *Ligophorus* on atypical hosts (Sarabeev et al. 2013).

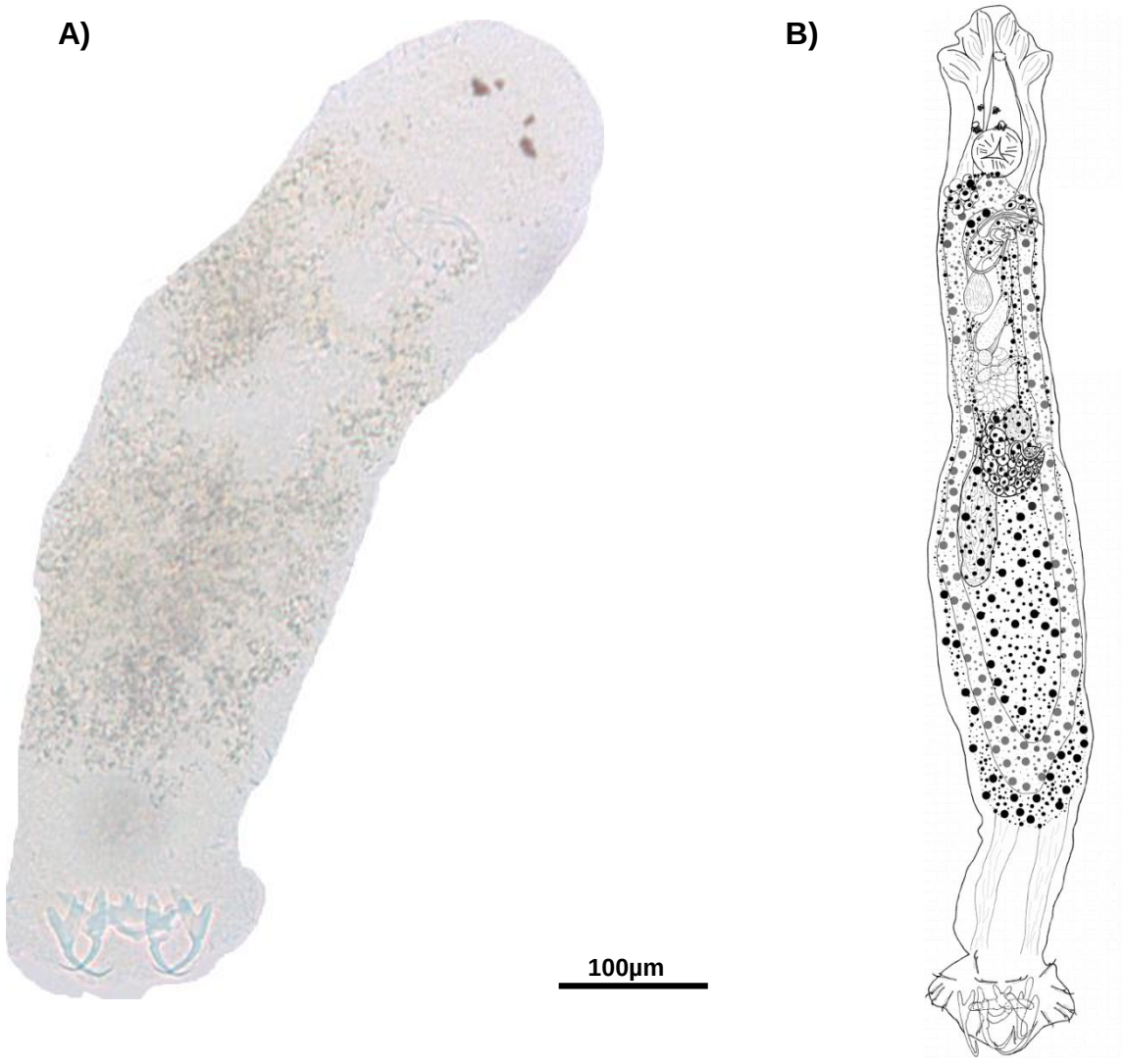


Figure 1.1. A) Photomicrograph of *Ligophorus cephali* and B) Drawing of *Ligophorus cephali* (from Rubtsova et al. 2009).

The genus is speciose, with some 60 valid species (Rodríguez-González et al. 2015b), and morphologically diverse, and well-resolved phylogenies are available (Blasco-Costa et al. 2012; Khang et al. 2016). *Ligophorus* and Mugilidae define an intriguing scenario of host-parasite

associations. At the species level, the salient pattern is that each species of *Ligophorus* predominantly occurs on a single host species and that often co-occurs with one or more congeneric species. In the Mediterranean and Black Seas, the 16 nominal species of *Ligophorus* known have been recorded on six grey mullet species (Blasco-Costa et al. 2012).

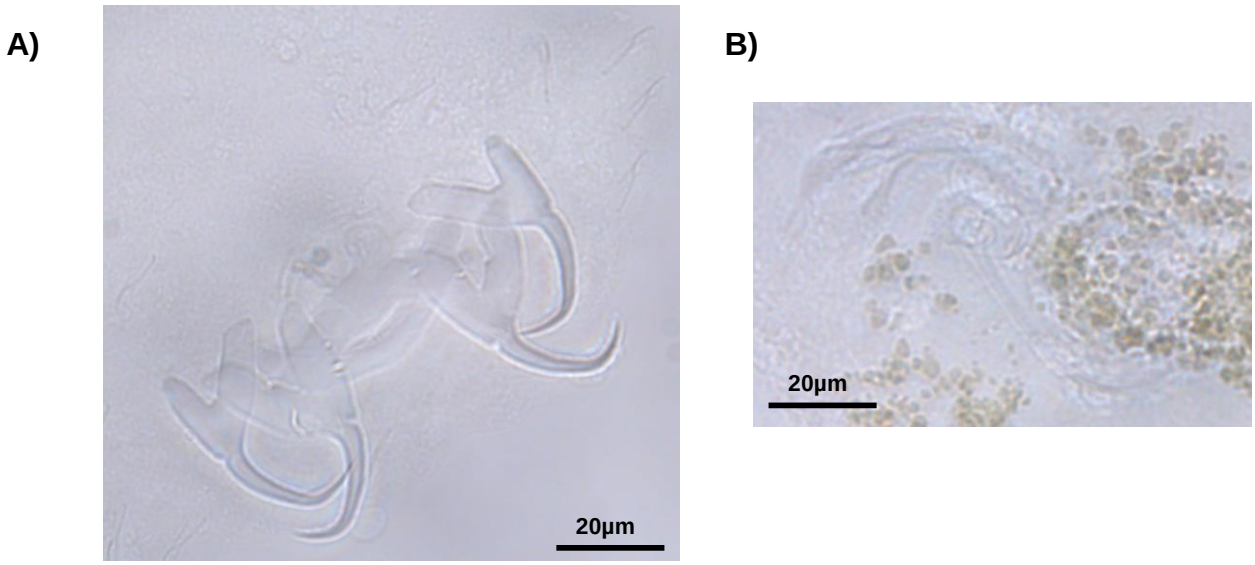


Figure 1.2. Micrographs of the sclerotized hard parts of *Ligophorus* sp. A) haptor and B) copulatory organ.

1.3. The hosts: Mugilidae

Grey mullets (Osteichthyes, Mugilidae) are a cosmopolitan family of teleost fishes occurring in most temperate, sub-tropical and tropical waters in both hemispheres (Cardona, 2006; Durand et al. 2012). They have an extraordinary adaptability, which has resulted in species that are found mainly in the clear and pristine waters of coral reefs to those that occur in highly turbid estuarine and freshwaters (Blaber, 2000). The Mugilidae encompass 17 genera and 72 species (Nelson, 2006) and represent an important fishery resource worldwide, and are successfully used in coastal aquaculture (Miranda-Filho et al. 2010).

In the brackish waters of the western Mediterranean, this family is represented by 6 species: *Chelon labrosus* (Risso, 1827), thicklip grey mullet; *Liza aurata* (Risso, 1810), golden grey mullet; *Liza*

ramada (Risso, 1826), thin-lipped grey mullet; *Liza saliens* (Risso, 1810), leaping mullet and *Mugil cephalus* Linnaeus, 1758, flathead mullet, *Oedalechilus labeo* (Cuvier, 1829), boxlip mullet; and *Liza haematocheila* (Temminck and Schlegel, 1845), so-iuy mullet. These species of mullets, except the little studied *O. labeo*, were chosen as model of study in this thesis (Figure 1.3).

The last species, *Lz. haematocheila* is native to the Amur River estuary and the Sea of Japan and was deliberately acclimated in the Black and the Azov Seas. This fish species established a successful reproductive population in the Azov Sea in the early 1980s (Sarabeev, 2015). The environmental conditions in the Black Sea and the Sea of Azov appear to be favourable to this species whose growth rate exceeds those of the native mullet species. Furthermore, Starushenko and Kazanski (1996) predicted its expansion towards the Mediterranean Sea, where it was reported in 1995. Along the shores of Black Sea, its expansion corresponds to a sharp decline of native species of Mugilidae, which it apparently displaces (Sarabeev, 2015).

A comprehensive review of parasites of grey mullets world-wide has been investigated mainly with parasites of the skin, gills and digestive tract (Merella and Garippa, 2001). Mullets offer an excellent scenario to study the geographical, ecological and evolutionary aspects of host-parasite associations. Current evidence suggest that this host-parasite system is characterized by a high degree of exchange of mullet parasites in areas such as the Mediterranean, Red Sea and Black Sea, where mullets are diverse and sympatric, and the presence of a number of local, strictly specific congeneric parasite species that are closely to these in adjacent areas (Blasco-Costa, 2009). Mullets harbour a diverse parasite fauna, which includes representatives of all major parasitic groups (protozoans, helminths and crustaceans).

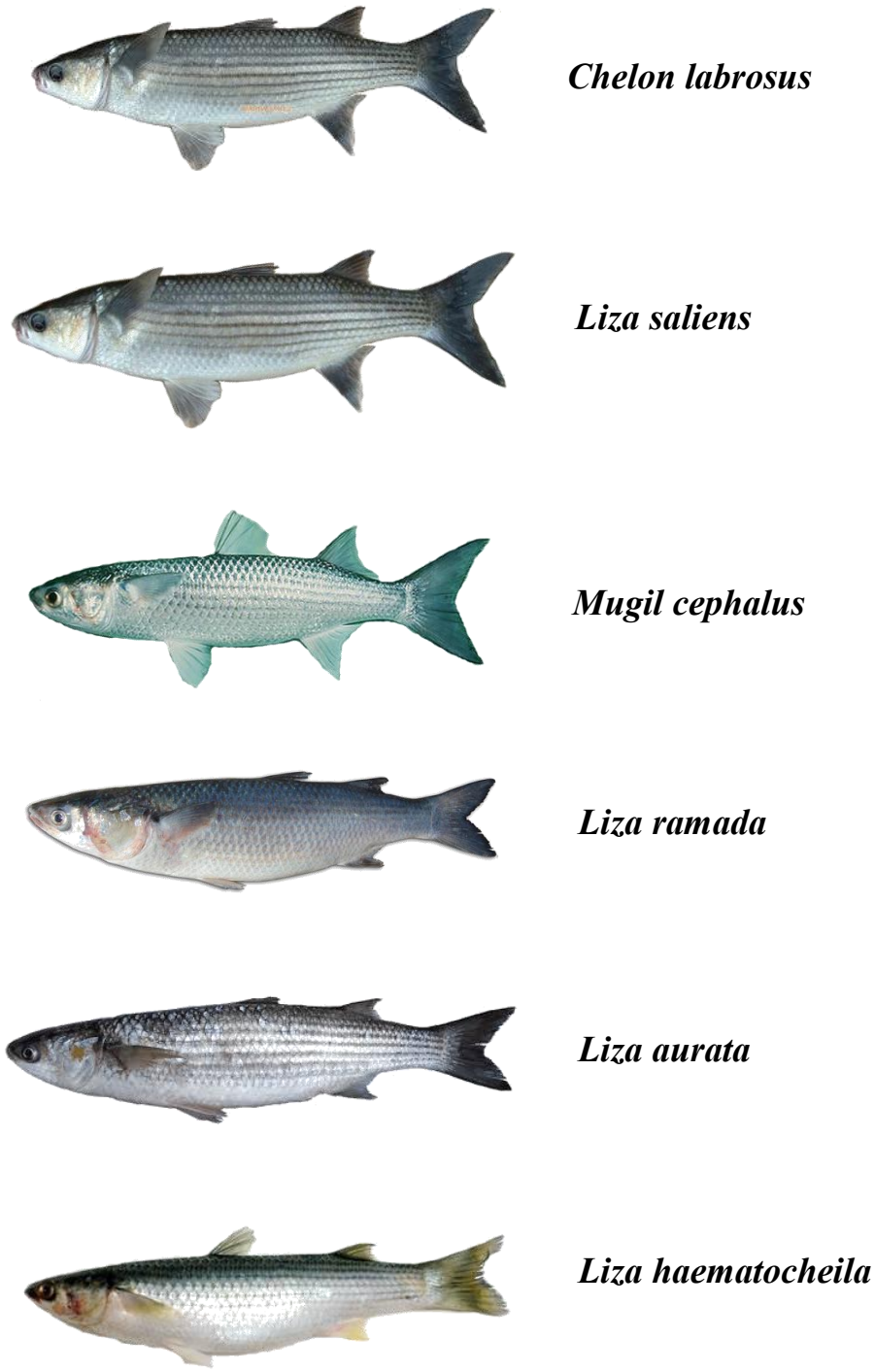


Figure 1.3. Host species of mullets analysed (Modified from http://www.fishbase.org/).

Records of *Ligophorus* outside the Mediterranean Sea are scarse. *Ligophorus mugilinus* (Hargis, 1995) has been reported from *M. cephalus* in the Gulf of Mexico and in the Caribbean on *Mugil curema* Valenciennes, 1836 (although there are doubts about the specific identity of the latter record) (Sarabeev et al. 2005). In other areas of the Americas, it also has been reported in different hosts: *Ligophorus huitrempe* Fernández-Bargiela, 1987, from *M. cephalus* in the Southern East Pacific (Fernández-Bargiela, 1987); *Ligophorus tainhae* Abdalah, Azevedo and Luque 2009, *Ligophorus brasiliensis* Abdalah, Azevedo and Luque, 2009, *Ligophorus guanduensis* Abdalah, Azevedo and Luque, 2009 and *Ligophorus lizae* Abdalah, Azevedo and Luque, 2009, *Ligophorus saladensis* Marcotegui and Martorelli, 2009 and *Ligophorus uruguayense* Failla and Otrowski de Núñez, 2009 in the Western Central Atlantic, on *Mugil liza* Valenciennes 1936 (syn. *Mugil platanus*) (Abdalah et al. 2009; Marcotegui and Martorelli, 2009; Failla and Otrowski de Núñez, 2009). In this thesis we describe new species of *Ligophorus* in the coastal waters of Yucatan, Mexico, and thus, contribute to the knowledge of the genus in the Americas.

Among the aforementioned *Ligophorus* in mullets, this study covered all grey mullet species reported as host of monogenean *Ligophorus* spp. in the Mediterranean, the Black Sea, and Sea of Azov and the new species recorded for America, *L. yucatanensis*.

1.4. Geometric morphometric on haptoral structures in Monogenea

In the Monogenea, the haptoral structures allow the attachment onto hosts. The morphology of these attachment structures has been studied in various ecological and evolutionary contexts (Vignon et al. 2011). So, the study of the phenotypic variability of the haptoral structures in *Ligophorus* offers an opportunity to investigate morphological variation over an evolutionary scale.

The morphometric variation in these structures has been studied using two approaches: traditional morphometrics (Marcus, 1990), which are not effective for capturing shape information

present in the geometry of defined points of a structure (Zelditch et al. 2012) (Figure 1.4), and geometric morphometrics. This method has revealed as excellent for extracting, visualizing and combining shape data with other data types such as molecular phylogenies to attain an integrative evolutionary analysis (Adams et al. 2013). Digitization of the anatomical structure of interest provides the key to the acquisition and use of a new type of data: landmarks coordinates, from which shape information can effectively be extracted, and then analyzed, using Procrustes superimposition, thin plate splines, relative warps analysis and other tools (Collyer et al. 2014). The current success of geometric morphometrics lays in the visualization framework provided, which can communicate even complex morphological changes much more efficiently than the tables of coefficients that result from traditional morphometric analyses (Klingenberg, 2013).

In the morphological analyses of monogeneans, the sclerotized parts and copulatory organ provide prominent morphological characters upon which their identification is largely based. Unlike other tissues, its hard parts are undistorted by preparative procedures and are an apparently reliable character on diagnoses (Vignon, 2011). Morphometric variation in all sclerotized parts of monogeneans has been studied for a long time from the perspective of systematics (Shinn et al. 2001) and evolutionary ecology (Mendlová and Šimková, 2014). Hard parts such as "anchors" are ideal model for geometric morphometric analysis because they are not easily deformed by compression when mounted onto slides (Lim and Gibson, 2009).

The analysis of monogenean morphometric data has been, and continues to be, dominated by the application of traditional morphometrics (Mariniello et al. 2004; Shinn et al. 2004; Soo and Lim, 2012). To date, there are only few studies (Vignon and Sasal, 2010; Vignon, 2011; Vignon et al. 2011; Llopis-Belenguer et al. 2015; Khang et al. 2016) that have applied geometric morphometrics to analyze monogenean anchor shape variation.

The paucity of geometric morphometrics studies, however, belies the importance of this approach in uncovering intra e interspecific shape variation in anchors that can be invaluable for

species delimitation, as well as for testing hypothesis of modularity and morphological integration between parts of anchors, evaluating levels of phenotypic plasticity and determine to which extent phenotypic similarity among species is the outcome of adaptive processes related to the ecology or the morphology of their fish host or a the reflection of phylogenetic constraints (Morand et al. 2002).

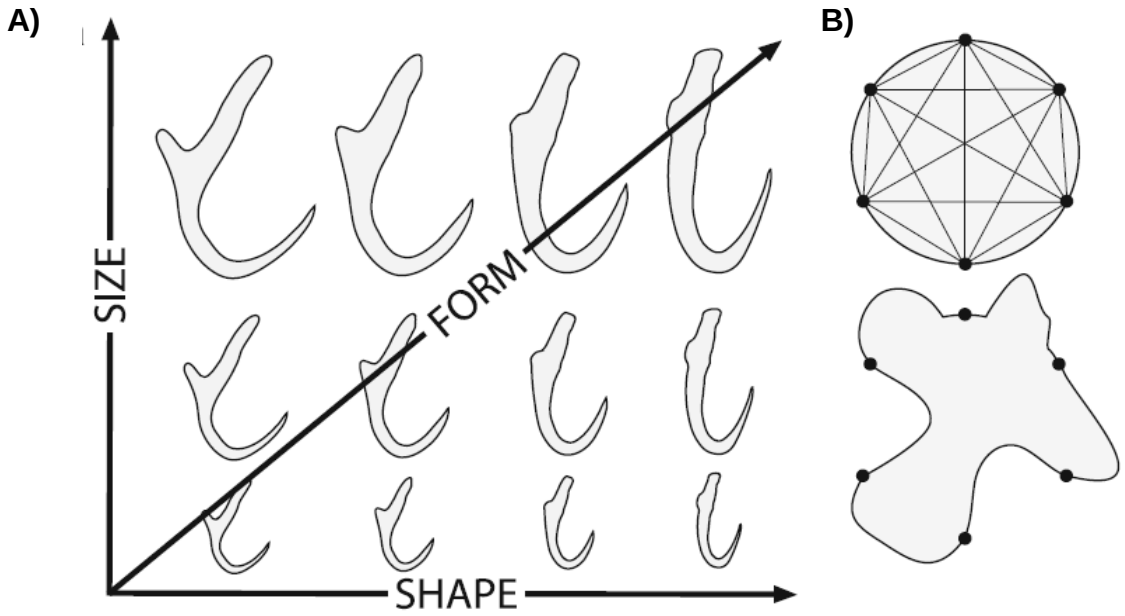


Figure 1.4. A) Change in 'form' consists of a combination of change in 'size' and 'shape' (morphological information independent from size, orientation and position in space). Whereas morphometrics involve the quantitative study of form, the measurements collected contain information pertaining to a combination of size and shape to various extents. B) The use of methods that maximise the amount of information (size and shape) from morphological features is not straightforward. In this example, the outline can provide information about the shape that cannot be captured by trusses (i.e. collection of linear distances between pairwise anatomical landmarks, black spots, distributed at equal intervals along the outline). Comparison of the sets of linear measurements collected from the two forms would suggest identical size and shape, whereas outline information shows clear differences in shape, with identical size (expressed as the surface of the grey area) (Taken from Vignon, 2011).

Moreover, analysis of patterns of congruence between the phylogenies of the grey mullets and their anchor morphometry of *Ligophorus* open the possibility to gain insight into prevalence of host

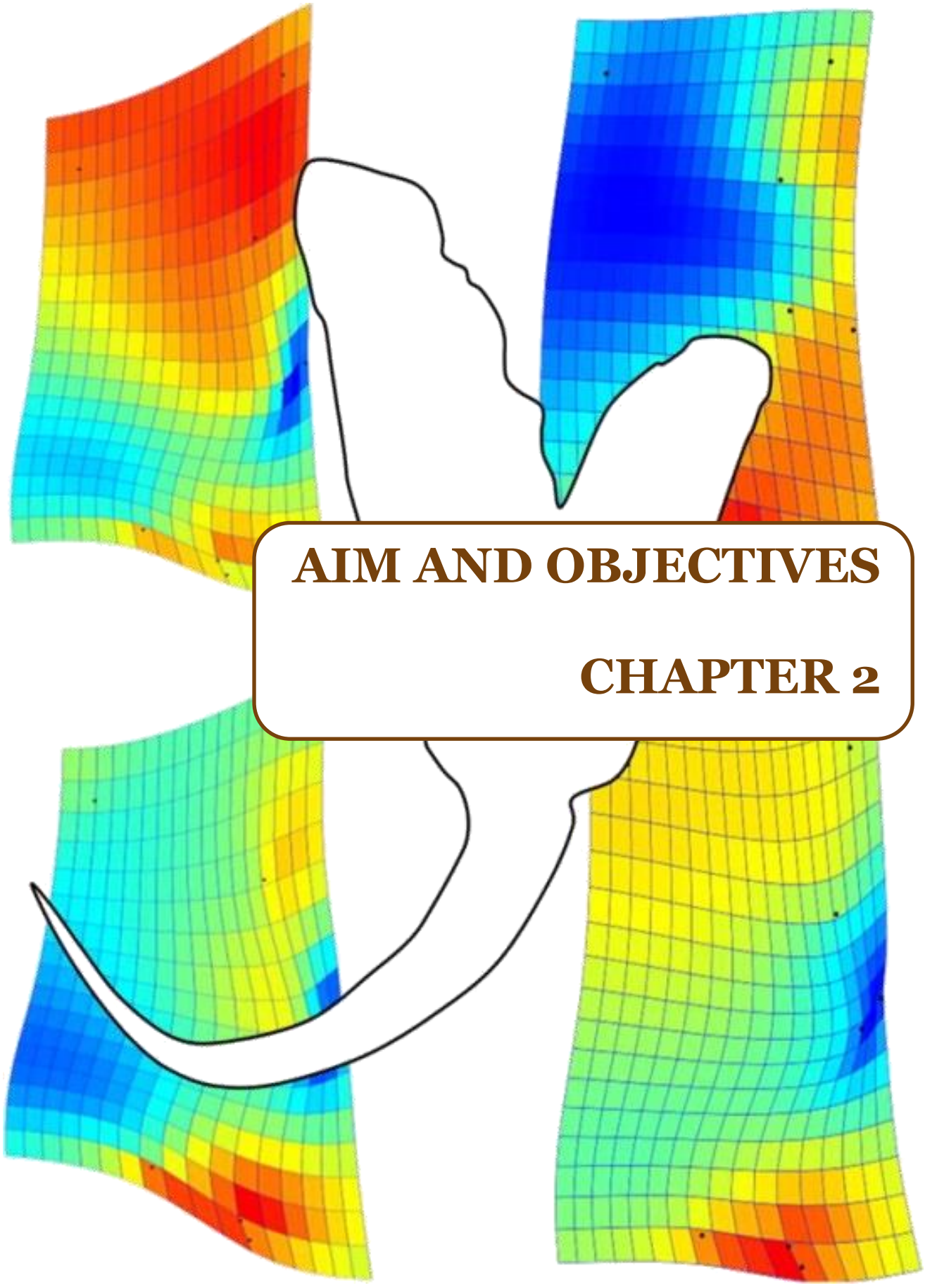
switching (Vanhove and Huyse, 2015) and thence assess the relative importance of allopatric and sympatric speciation in shaping the diversity of this genus.

1.5. This study

This study has been carried out under support of National Plan for Scientific Research, Development and Technological Innovation of Spain (CGL2008-02701) the Generalitat Valenciana, Spain (Prometeo Project 2015/018), Ministry of Economy and Competitivity, Spain (CGL2015-71146), and from Consejo Nacional de Ciencia y Tecnología (CONACYT-CONCYTEY) of the Government and Yucatán State, México (No. 204397).

This thesis is devoted to studying the phenotypic variability in shape and size of the haptoral structures of 14 species of *Ligophorus* from 6 grey mullets using geometric morphometrics to provide new and previously unexplored insights into the functional morphology of attachment onto hosts and evolutionary processes of host-parasite coevolution, as well as to increase knowledge of the zoogeography and biodiversity species of the genus by addressing the following questions:

i. What are the patterns of anchors shape and size variation in relation to site attachment?
ii. Which roles play the root and points of anchors in *Ligophorus* spp. play in attachment?
iii. Is there sufficient morphological evidence to describe of a new species of *Ligophorus* in the Americas?
iv. How is the evolution of anchor morphology in the species of *Ligophorus*? Are characters independent or modular?
v. Is there a phylogenetic signal in the shape and size of *Ligophorus* spp.?



AIM AND OBJECTIVES

CHAPTER 2

Aim

In the present study, we applied geometric morphometrics to explore intra e interspecific morphological variations in haptoral structures in species of *Ligophorus*, as well as to determine relationships of parasite phylogeny with anchor form, to eventually underpin relationships between functional morphology of parasite attachment to the host and evolutionary processes.

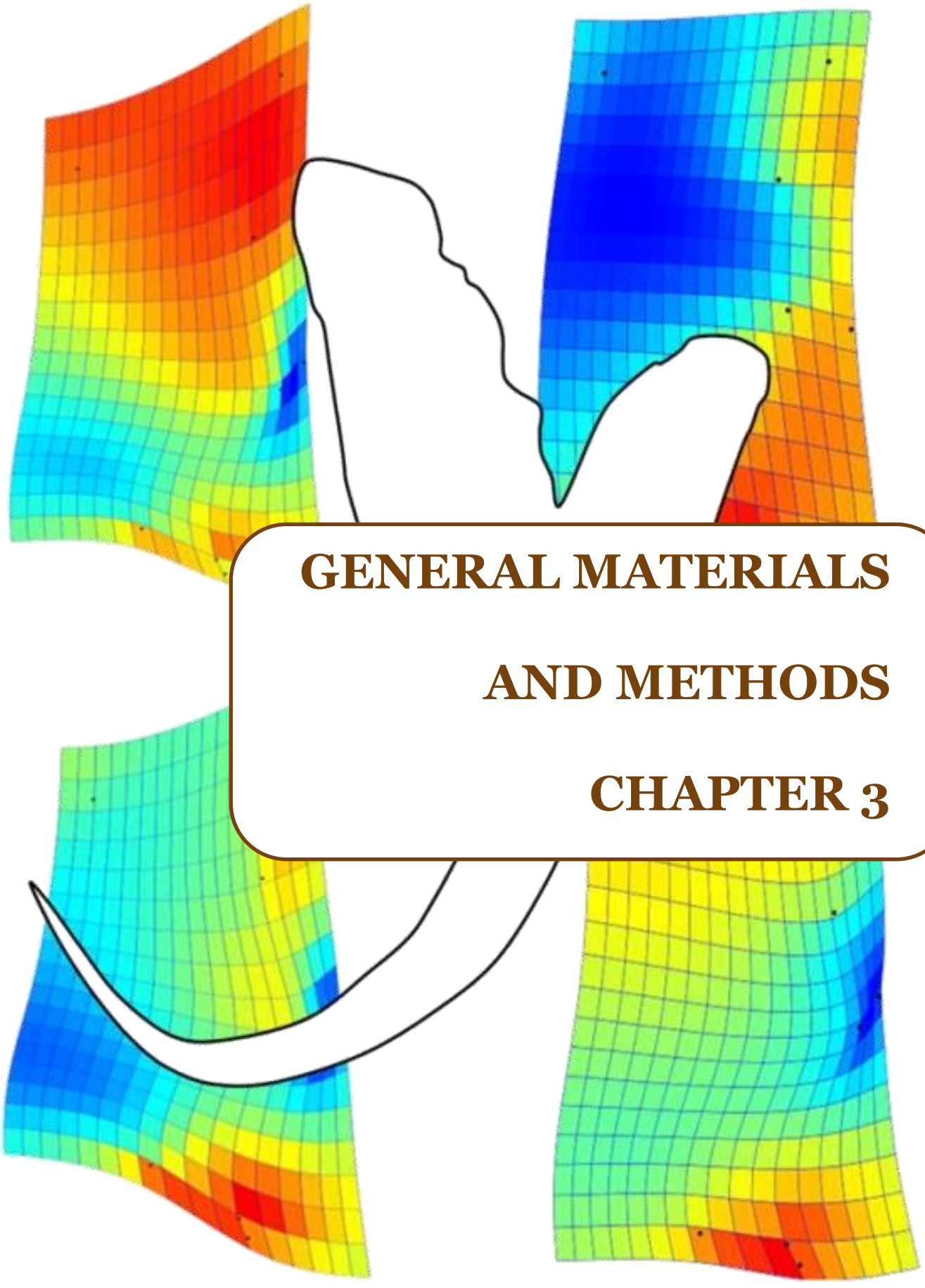
Objectives

- To analyze the variability in shape and size of the dorsal and ventral anchors of *Ligophorus cephali* from *Mugil cephalus* by means of geometric morphometrics and multivariate statistics and to assess the morphological integration between anchors and between roots and points.

- To describe a new species of *Ligophorus* from *M. cephalus* from the Yucatán Peninsula, Mexico and to update the zoogeography of *Ligophorus* spp. in the light of current evidence for a complex of cryptic host species under the denomination of *M. cephalus*.

- To determine whether variation in anchor shape in 14 *Ligophorus* spp. is modular and integrated by testing four hypotheses of modularity at both morphological and evolutionary levels.

- To assess phylogenetic signal in the form of ventral and dorsal anchors of 14 species of *Ligophorus* on mullets from the Mediterranean, Black Sea and Sea of Azov in order to evaluate whether the similarity in form is due to convergence or shared evolutionary history.



GENERAL MATERIALS AND METHODS

CHAPTER 3

In this section we provide a brief description of the different materials and methods used in this study. Detailed methodology will be explained in the corresponding chapters.

3.1. Study area and fish sampling

The grey mullets (Mugilidae) were sampled at six localities. Three of them were in the Spanish western Mediterranean: the Ebro Delta (40°30'-40°50'N, 0°30'-1°10'E), Santa Pola Bay (38°00'-38°20'N, 0°10'-0°40'W); and L'Albufera, a coastal lagoon (39°20'0"N-0°21'0"W). Additionally samples were taken in the Kerch Strait, Sea of Azov (45°16'20.8"N-36°31'40.6"E) and the Artemovka Delta, Sea of Japan (43°18'30.3"N-132°17'4.8"E). Fishes were also collected in Celestún, Yucatan Mexico (20°51'33"N-90°24'00"W) (Figure 3.1).

The fish hosts collected for this thesis were purchased from local fisherman at local fish markets from the six localities during the spring (2004), autumn (2005), spring-autumn (2011) and spring (2014) (See details in the respective chapter). Grey mullets are locally and globally abundant and are not subject to special conservation regulations in Spain, Russia and Ukraine and the species involved are listed by the IUCN as "Least Concern".

Collections of fish species differed among sites and seasons both in number and range due to collecting opportunity and differences in local fish fauna. Six species of mullets were examined: the flathead mullet *M. cephalus*; the so-iuy mullet *Lz. haematocheila*; the golden grey mullet *Lz. aurata*; the thinlip mullet *Lz. ramada*; the leaping mullet *Lz. saliens* and the thicklip grey mullet *C. labrosus* (see Figure 1.3). Fishes were surveyed for parasites within a day of their capture or after freezing. The number of fishes used in this study was 31 in chapter 4, 13 in chapter 5, and 77 in chapters 6 and 7. See more details in theses chapters.

Fishes from Mediterranean Sea were killed, immediately frozen. The total length and weight were recorded for each species of fishes.

3.2. Parasite collection and morphological study

Specimens belonging to *Ligophorus* were recovered from different species of mullets in this study (See details in chapters 4 to 7). The gills from each host species were removed and their surface was individually examined for monogeneans of *Ligophorus* spp. Some gills were examined in fresh immediately transported to the laboratory in order to collect live material for the morphological, geometric morphometrics and DNA isolation. The remaining fishes were frozen at -20°C and examined at a later stage when all parasites were collected, identified and counted.

The gills were surveyed under a stereomicroscope. Infected gills were then fixed in a plastic container with 4% formalin for 3-4 hours to keep the monogeneans attached at their specific sites in the gills, before being stored in 70% alcohol (Rodríguez-González et al. 2015a).

3.2.1. Morphological analyses

For the morphometric and geometric analyses, an enzymatic digestion technique was used to improve visualization of the sclerotized structures in each species of *Ligophorus* (Paladini et al. 2011; see details in chapter 4). The specimens were identified on the basis of morphological traits of these structures based on Rubtsova et al. (2006), Dmitrieva et al. (2009) and Sarabeev et al. (2013).

After digestion, most specimens were mounted directly in glycerin jelly (Sarabeev et al. 2013) and some were preserved in 70% alcohol, stained in iron acetocarmine, dehydrated through an ethanol series (from 70 to 100%), cleared in dimethyl phthalate and mounted as whole mounts in Canada balsam to ascertain details of their soft internal anatomy. This mounting technique only was used for taxonomy in chapter 5.

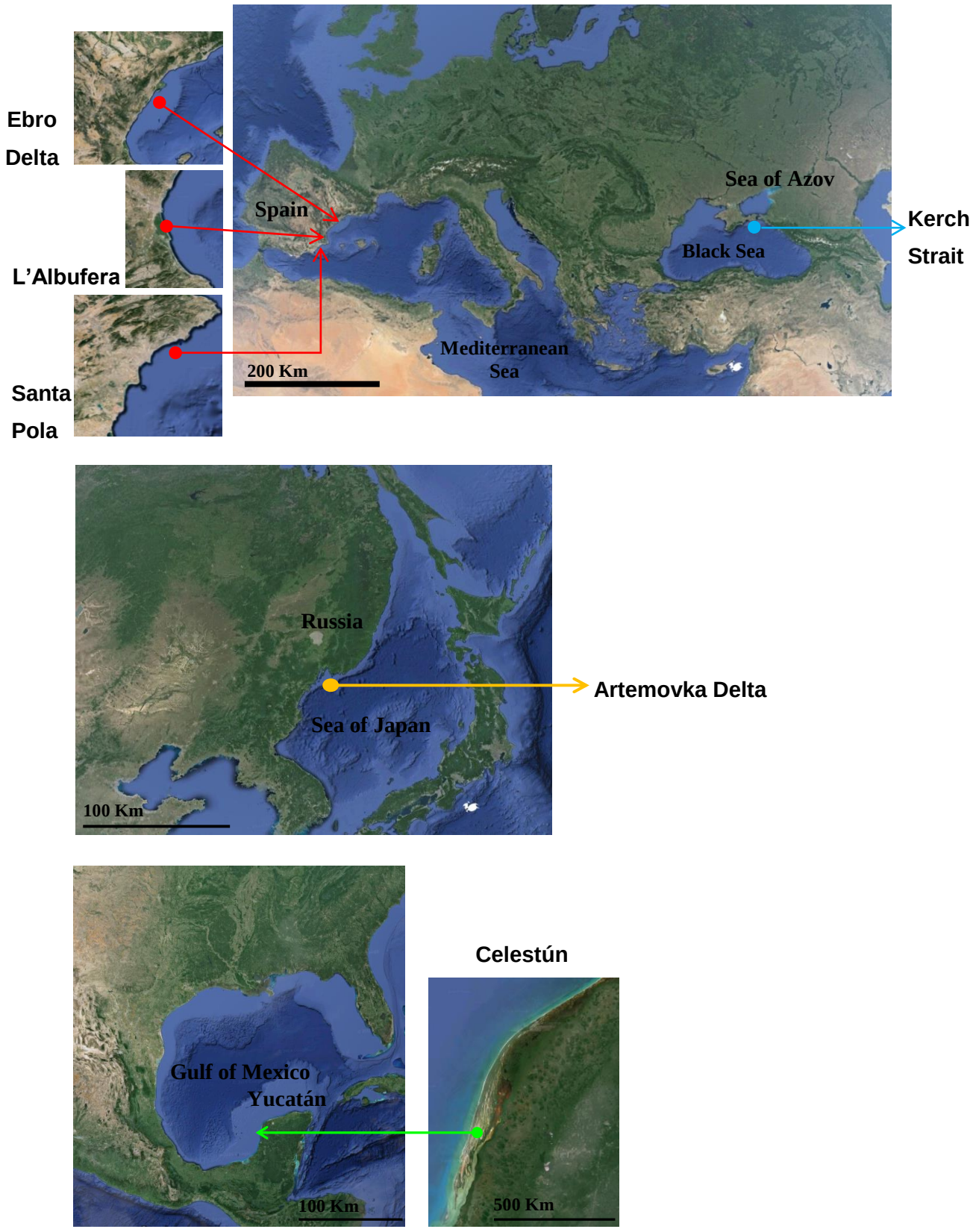


Figure 3.1. Locations map of the main sampling sites included within this study (Taken from https://earth.google.com).

In the laboratory, the gills and specimens of *Ligophorus* were examined under a stereoscopic microscope or with light microscope (LEICA DMR) with the aid of differential interference contrast for indentification and detailed morphological examination. Measurements are given in micrometers as mean ± standard deviation (SD) followed by ranges in parentheses (Details see in chapter 5).

3.3. Geometric morphometrics analyses

3.3.1. Processing of materials for geometric morphometrics

For this thesis, only the anchors (ventral and dorsal) from each adult specimens on both sides were considered for geometric morphometrics techniques because they are not subject to large variation due to contraction or flattening on fixation (Lim and Gibson, 2009). Under this approach, the bars were not studied because they are more difficult to observe flat and are more prone to distortion during fixation and mounting (Vignon and Sasal, 2010). Specifically, one anchors from each pair (left or right) in each specimen for species of *Ligophorus* was chosen. The anchors were drawn using a drawing tube at 100× magnification (under immersion oil) in a Nikon Optiphot-2 microscope equipped with interference contrast. Photographs were taken when necessary (see details in chapters 6 and 7) under an interference contrast and with a Leica DC150 digital camera.

3.3.2. Acquiring landmark data

This study was based on a two-dimensional (2D) Cartesian landmark coordinates geometric morphometric approach (Zelditch et al. 2012). This technique allows analyzing separately the two components of variation of form: size and shape, and visualizing the results as shape changes of specific regions of the biological structures under examination. Raw images of the anchors of all specimens of *Ligophorus* were compiled and scaled with tpsUtil version 1.52 (Rohlf, 2012).

In this study, we have chosen 8 homologous landmarks for all species of *Ligophorus* evaluated (see chapters 4, 6, 7). Landmarks are points of correspondence on each specimen that match between and within species or, equivalently, biologically homologous anatomical loci recognizable on all specimens. In addition, the landmark configurations were selected to offer an adequate summary of the anchor morphology in *Ligophorus* spp. So, the composition of landmarks chosen represented a complete coverage of the structure. Moreover, our landmarks were digitizable (consistently replicable with a high degree of accuracy). Finally, the 2D data landmarks were coplanar and conserved the topological positions relative to other landmarks. An example of such landmarks can be visualized in Figure 3.2 using *L. cephali* as model. Landmarks were always digitized in the same order. Then, two-dimensional landmark coordinates were extracted from scanned images using the free software tpsDig version 2.17 (Rohlf, 2013).

3.3.3. Extracting shape information: the superimposition methods

The principal and most important analysis of geometric morphometrics is called Procrustes superimposition (GPA) (Klingenberg, 2010), where only the shape information is extracted and the other components of variation in size, position and orientation are removed, while taking care not to alter shape in any step of the procedure (Goodall, 1991; Rohlf, 1999). Thus, for each species of *Ligophorus*, and for both ventral and dorsal anchors, the extra components of variation were removed by rescaling the configurations to a standard size, shifting them to a standard position, and rotating them to a standard orientation (Bookstein, 1996) (Figure 3.3).

Centroid size (CS) under this approach is quantified as a measure of size, and is computed as the square root of the sum of squared distances of landmarks from the centre of gravity of a configuration (Zelditch et al. 2012).

The resulting analysis of Generalized Procrustes Analysis (GPA) produced a matrix of shape coordinates for subsequent analyses (see chapters 4, 6 and 7). Because this variation concerns the

relative displacements of landmarks to each other in many directions, we used multivariate methods (Klingenberg, 2010). Accordingly, these multivariate analyses simultaneously consider the covariation of all landmark coordinates. Most of them find new variables, corresponding to directions in shape space. For instance, principal component analysis can be used to examine the main patterns of variation in the data, multivariate regression can be used for analysing allometry or evolutionary change in shape over time, partial least squares analysis can be used to examine covariation of shapes, etc. A wide range of additional multivariate methods exist, some of which have been specifically devised for morphometric applications (Klingenberg, 2010).

Shape variation was characterized in shape spaces. A shape space represents all possible shapes for a given number of landmarks by points, so the distances between points represents the similarities between the corresponding shapes (Klingenberg, 2010). The shape changes were visualized by the thin-plate spline technique (interpolate shape changes from the landmarks to rectangular grids) and outline drawings (Klingenberg, 2013).

The analyses of geometric morphometric in this study were performed with MorphoJ version 1.06d (Klingenberg, 2011).

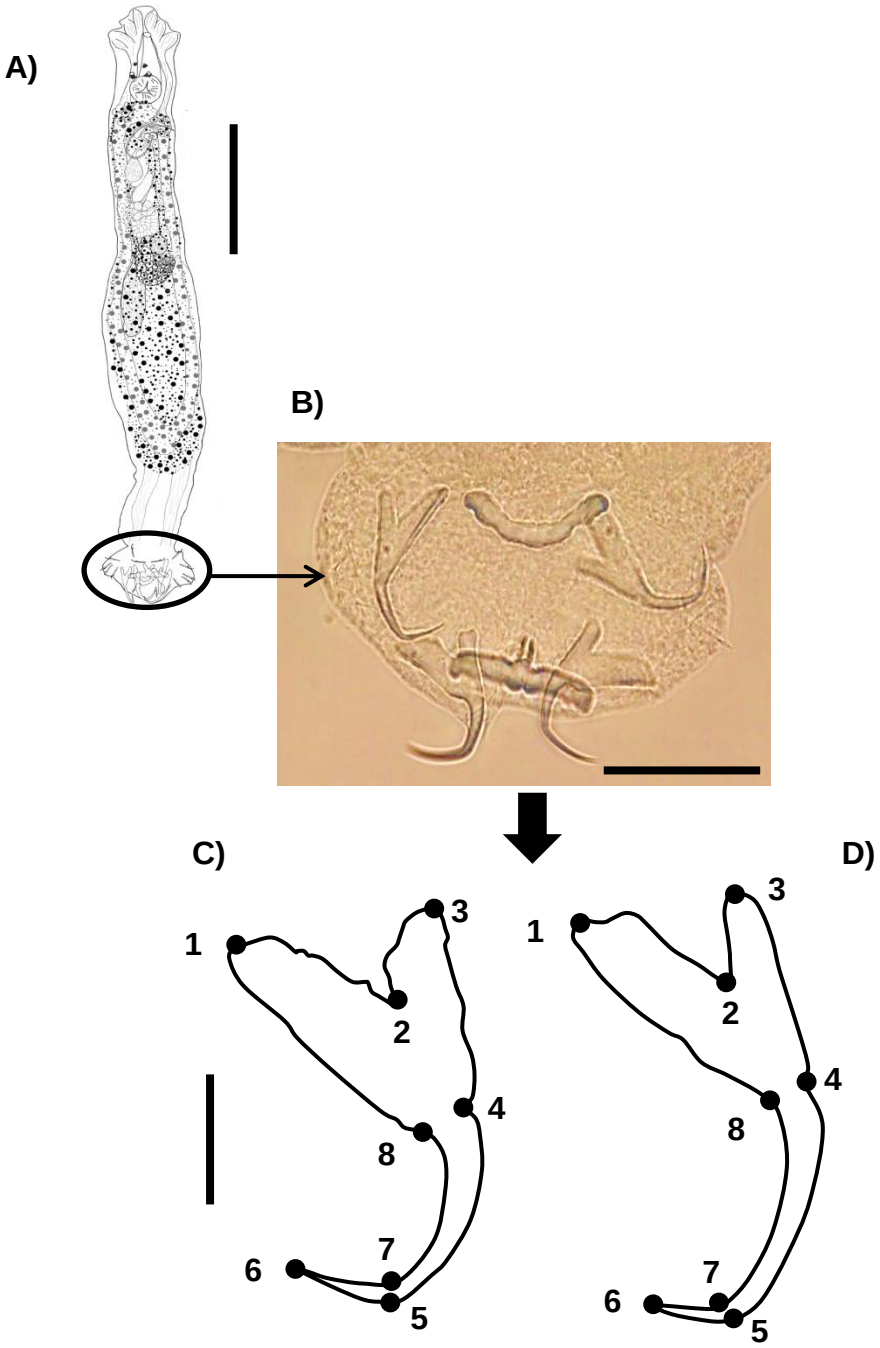


Figure 3.2. A) *Ligophorus cephali* Rubtsova, Balbuena, Sarabeev, Blasco-Costa et Euzet, 2006. B) Micrograph of haptoral sclerotized structures. Ventral C) and Dorsal D) anchors of *L. cephali* (drawings). The positions of eight landmarks were used for morphological analyses: 1) maximum point of inner root, 2) inflection between outer and inner root, 3) mean point of outer root, 4) outer shaft base, 5) outer point base, 6) anchor point, 7) inner point base and 8) inner shaft base. Scale bars= A) 100, B) 40 and C) 100 mμ.

3.3.4 Allometry and size correction

Allometry refers to the dependence of shape on size and tends to be one of the dominant factors of morphological variations, reflecting the abundant variation of size (Klingenberg, 2016). Allometry was evaluated in our dataset because size variation can affect the entire structure. We used a multivariate regression of Procrustes coordinates (as shape variables) against log-transformed centroid size (as size variables) to eliminate the effect of size on shape. Regression fits a straight line to the data points that represent the expected shape for each value of size. The deviations of individual data point from this line – the residuals – represent shape variation that is not explained by size. A correction for the effects of allometry was implemented by using these residuals from the regressions of anchor shape on anchor size in further analyses (Klingenberg and Marugán-Lobón, 2013) (details in chapters 6 and 7).

3.3.5 Phylogenetic analyses and shape onto phylogeny

To obtain a phylogeny of the species of *Ligophorus*, the 28S rDNA and ITS1 sequences of the Mediterranean (Blasco-Costa et al. 2012) were used in the materials and methods section of the respective chapter. The sequences of each gene were aligned using MUSCLE (Edgar, 2004) (Tamura et al. 2013). The alignments of 28S and ITS1 sequences comprised 866 and 779 positions, respectively. For phylogenetic reconstruction, the nucleotide substitution model best fitting the sequences was estimated independently for each dataset using jModelTest (Darriba et al. 2012). The model eventually selected in each case is specified in the materials and methods section of chapter 6.

The aligned sequences from the two genes were concatenated. We used both Maximum Likelihood (ML) and Bayesian Inference (BI) for phylogentic reconstruction. For ML, a starting tree was built based on neighbor joining. Branch support was estimated by bootstrap analysis with 1000 replicates.

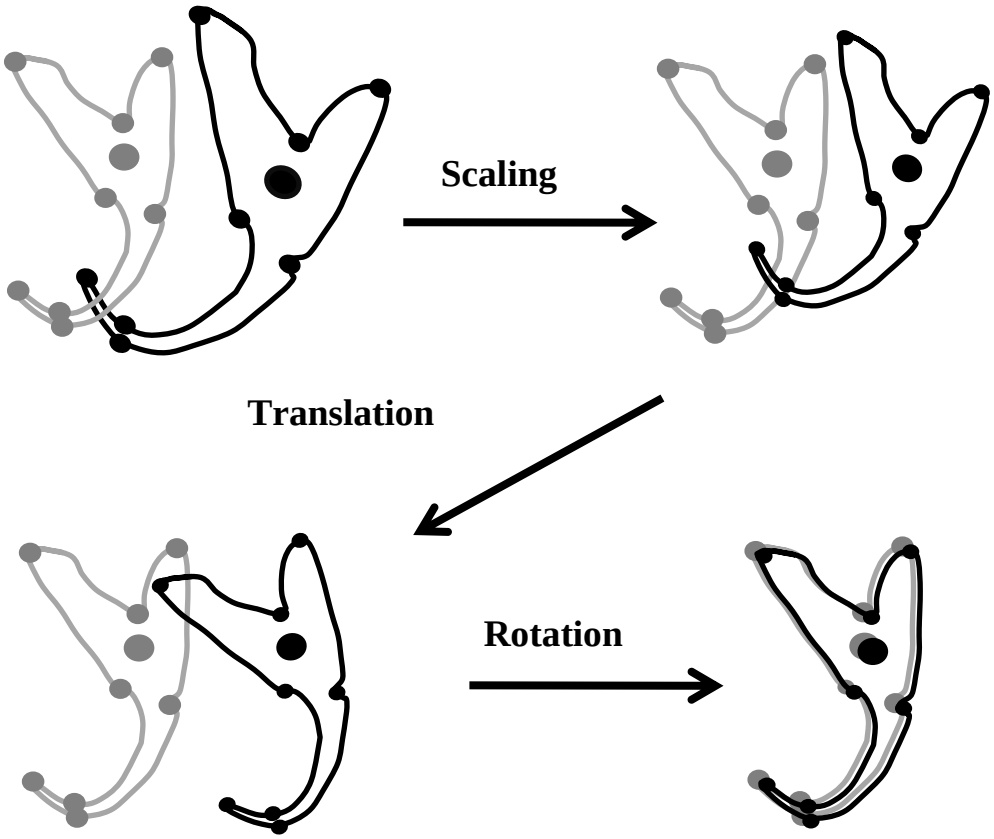


Figure 3.3. Summary of Procrustes Superimposition Analysis (GPA) of two hypothetical anchors. Components of variation other than shape are eliminated by scaling to the same size, translating to the same location of centroids, and rotation to an overall best fit of corresponding landmarks (small points). Centroid size is computed as the Euclidean distance of each landmark to the centre of gravity (large points) of each configuration.

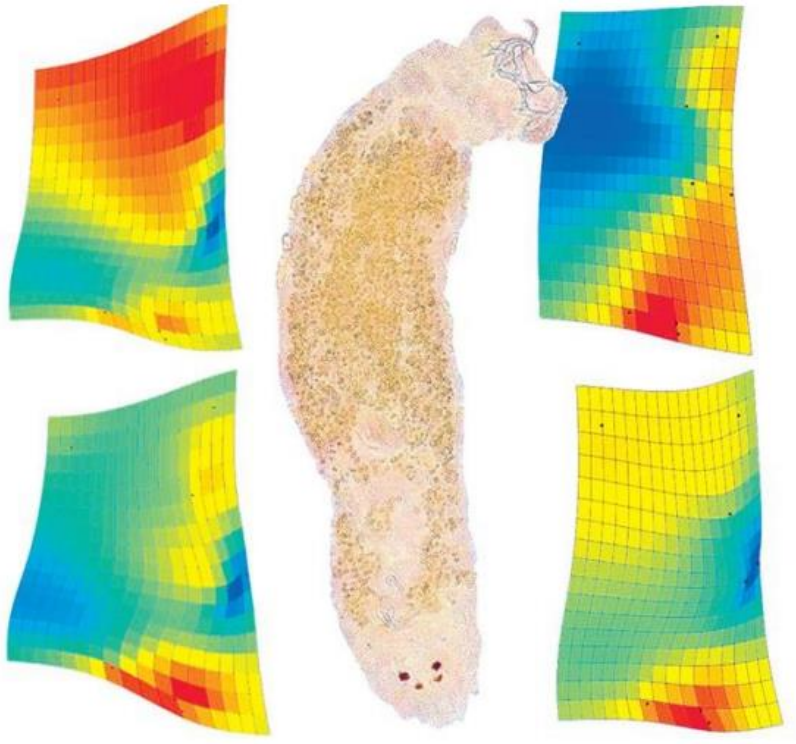
The BI analysis was performed with four Markov chain Monte Carlo ran for 10^6 generations with a sampling frequency of 1,000 an a "burn-in" set value of 25% of the stored trees. Nodal support was estimated as posterior probabilities (Huelsenbeck et al. 2001). More details are given in chapter 6.

Since the Bayesian and Maximum Likelihood trees obtained were very similar, we used only the former for projection onto the shape and size morphospaces. This was mapped on the shape (Procrustes coordinates) and size (Log-centroid size) morphospaces estimated by squared-change parsimony, assuming a Brownian motion model of evolution to reconstruct the ancestral states of shapes at internal nodes of the phylogeny (Madisson, 1991), which was weighted by genetic change

on the respective branches of the tree .This analysis was performed in the package MorphoJ 1.06d (Klingenberg, 2011). By means of these calculations, the sum of squared changes of shape along the branches is minimized over the entire phylogeny. We used a permutation approach (Klingenberg and Gidaszewski, 2010; Klingenberg and Marugán-Lobón, 2013), which simulated the null hypothesis of no phylogenetic signal in the data. Furthermore, the significance of phylogenetic signal was established by 10,000 random permutations and the total amount of squared change summed over all branches of the tree were conducted. To visualize the phylogenetic history of shape and size change, the tree was mapped onto principal component plots. The previous analyses provided values of tree length that are inversely related to the strength of the correlation between shape or size and phylogeny (Klingenberg and Gidaszewski, 2010) (specific details are given in chapter 7).

CHAPTER 4

Phenotypic plasticity in haptoral structures of *Ligophorus cephali* (Monogenea: Dactylogyridae) on the flathead mullet (*Mugil cephalus*): a geometric morphometric approach



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Abstract

Evaluating phenotypic plasticity in attachment organs of parasites can provide information on the capacity to colonise new hosts and illuminate evolutionary processes driving host specificity. We analysed the variability in shape and size of the dorsal and ventral anchors of *Ligophorus cephali* from *Mugil cephalus* by means of geometric morphometrics and multivariate statistics. We also assessed the morphological integration between anchors and between the roots and points in order to gain insight into their functional morphology. Dorsal and ventral anchors showed a similar gradient of overall shape variation, but the amount of localised changes was much higher in the former. Statistical models describing variations in shape and size revealed clear differences between anchors. The dorsal anchor/bar complex seems more mobile than the ventral one in *Ligophorus*, and these differences may reflect different functional roles in attachment to the gills. The lower residual variation associated with the ventral anchor models suggests a tighter control of their shape and size, perhaps because these anchors seem to be responsible for firmer attachment and their size and shape would allow more effective responses to characteristics of the microenvironment within the individual host. Despite these putative functional differences, the high level of morphological integration indicates a concerted action between anchors. In addition, we found a slight, although significant, morphological integration between roots and points in both anchors, which suggests that a large fraction of the observed phenotypic variation does not compromise the functional role of anchors as levers. Given the low level of genetic variation in our sample, it is likely that much of the morphological variation reflects host-driven plastic responses. This supports the hypothesis of monogenean specificity through host-switching and rapid speciation. The present study demonstrates the potential of geometric morphometrics to provide new and previously unexplored insights into the functional morphology of attachment and evolutionary processes of host–parasite coevolution.

Key words: Geometric morphometrics, Phenotypic plasticity, Haptor, Monogenean, Western Mediterranean, Mugilidae.

4.1. Introduction

Establishing the determinants of host specificity in parasites has both theoretical and applied implications. The former pertain to the study of evolutionary patterns between hosts and parasites and revolve around a central problem in evolutionary ecology (Gemmill et al. 2000): when does natural selection favour the evolution of specialists over generalists? On the applied side, delineating the host range of a given parasite is fundamental for both the design and implementation of control strategies (Murphy, 1998), and the evaluation and forecast of the impact of parasites associated with host introductions (Woolhouse et al. 2005).

Classically, the specificity of a host-parasite system is commonly believed to be the result of an adaptive process (Brooks and McLennan, 1991) and it has been suggested that high degrees of host specificity might be explained by the tight coevolutionary interaction between hosts and parasites (Poulin, 1992). Thus parasites would tend to optimise exploitation by adapting locally to the environment provided by their hosts and developing specific morphological, physiological and behavioural traits (Bush, 2009). However, other evolutionary processes might also lead to tight host specificity. Desdevises (2007) proposed that host switching could be a major driver of host specificity in some parasites such as monogeneans and particularly in marine systems. Under such scenario, phenotypic variability could increase the spectrum of hosts available; this provides switching opportunities which, coupled with rapid speciation by parasites, could account for high host specificity, as frequently observed in marine monogeneans (Desdevises, 2007).

Many monogeneans are characterised as being highly specific, restricted to certain gill arches and certain parts of gill filaments, and having developed different strategies in adapting to this microhabitat (Whittington and Kearn, 1991; Vignon et al. 2011). This adaptive process suggests that the high morphological variability of attachment organs in monogeneans is possibly linked to host specificity (Morand et al. 2002). Thus the evaluation of phenotypic plasticity of the organs responsible for attachment to the gills can inform us on the capacity to colonise new hosts and

would eventually cast light on evolutionary forces driving host specificity in monogeneans and other parasites in general (Poisot and Desdevises, 2010).

Despite this, few studies have focused on this topic (i.e., Olstad et al. 2009; Mladineo et al. 2013). Caltran et al. (1995a, b) observed that populations of *Ligophorus imitans* Euzet and Suriano, 1977 from *Liza ramada* Risso, 1827 display high morphological and anatomical variability of haptoral structures and genitalia, and revealed that variations in these organs are independent of each other. This variability was higher than that originally described by Euzet and Suriano (1977) for the other *Ligophorus* spp., but similar to that observed in *Dactylogyrus* (Dactylogyridae) and *Diplectanum* (Diplectanidae) (Belova, 1988; Silan and Maillard, 1989). In addition, the evaluation of environmental and demographic variables in morphological plasticity was reflected in the correlation between the size of haptoral anchors and host size, which the authors related to an increase in gill heterogeneity in larger fish.

These studies, similar to most others to date (except Olstad et al. 2009), have been based on linear measurements. The problem with this approach is that the pure shape information is frequently not obtained, making it impossible to partition size and shape for separate analyses (Corti et al. 2001). Geometric morphometrics can address this issue effectively and, in addition provide visualisation tools to better appreciate morphological variability (Bastir and Rosas, 2005; Vignon and Sasal, 2010; Zelditch et al. 2012). This technique has been successfully utilised in monogeneans to study ecological and evolutionary questions (Vignon and Sasal, 2010; Vignon et al. 2011), including phenotypic plasticity in *Gyrodactylus* spp. (Olstad et al. 2009).

We adopted this approach herein to examine the intraspecific variability and phenotypic plasticity of the ventral and dorsal anchors of *Ligophorus cephali* Rubtsova, Balbuena, Sarabeev, Blasco-Costa and Euzet, 2006 on the gills of *Mugil cephalus* L., 1758. Our focus was on the dorsal and ventral anchors as structures primarily responsible for attachment to the host gills. Specifically, we (i) describe, quantify and test patterns of shape and size variation in relation to site attachment on

the host individual, and (ii) evaluate the morphological integration between ventral and dorsal anchors, and between the roots and points of anchors, in order to gain insight into their functional morphology.

4.2. Materials and methods

4.2.1. Study site, host and parasite collection

Flathead grey mullets (*M. cephalus*) were collected in L'Albufera, Spain (39°20'N-0°21' W), in April-May 2011. L'Albufera is a 23.2 km2, shallow, eutrophied, Mediterranean lagoon surrounded by marshlands mainly devoted to rice crops, orchards, scattered country houses and coastline resorts (Soria et al. 2000; Soria, 2006). Fishes (n= 31) were purchased from local fishermen and were immediately transported to the laboratory for examination. Their total length ($\bar{X}$ ± S.D.: 32.5 ± 3.5 cm) and weight (404.2 ± 130.5 g) were recorded.

The gills were surveyed for monogeneans under a stereomicroscope on the day of capture. Infected gills were then fixed in a plastic container with 4% formalin for 3-4 h to keep the monogeneans attached at their sites before being stored in 70% alcohol (Rubio- Godoy, 2008).

For the morphometric analyses, an enzymatic digestion technique was used to obtain the sclerotized structures. A mixture of 300 µl of TE9 buffer (500 mM Tris-HCl, 200 mM EDTA, 10 mM NaCl, pH 9) and 100-200 µl of proteinase K (10 mg/ml) was used (Mo and Appleby, 1990; Paladini et al. 2011). Slides were then mounted in Kaiser's glycerol-gelatin and examined under a microscope at 100 x magnification. The specimens were identified as *L. cephali* on the basis of morphological traits (haptoral and copulatory structures) based on Rubtsova et al. (2006), Dmitrieva et al. (2009) and Sarabeev et al. (2013).

Only the anchors (i.e., ventral and dorsal, from each specimen) on both sides were considered for geometric morphometric techniques because they are not subject to large variation due to contraction or flattening on fixation (Lim and Gibson, 2009). The bars were not studied because

they are more difficult to observe flat and more prone to distortion during fixation and mounting (Vignon and Sasal, 2010). Specifically, one anchor from each pair (left or right) from each different specimen was chosen for analysis. Thus, the differences between the right and left side of each pair of ventral and dorsal anchors were not assessed.

The anchors were drawn using a drawing tube at 100 x (under immersion oil) under a Nikon Optiphot-2 microscope equipped with interference contrast.

4.2.2. Molecular data

Evaluating phenotypic plasticity requires assessment of the degree of genetic variation in the sample. To this end, we sequenced and compared the internal transcribed spacer 1 region (ITS1) of rDNA. Ten specimens were unmounted and transferred into 200 µl of TE9 buffer (500 mM Tris–HCl, 200 mM EDTA, 10 mM NaCl, pH 9) (Wu et al. 2007) to clean the glycerol-gelatin from the specimens. The DNA was extracted using an Qiagen DNeasy® Blood & Tissue Kit following the manufacturer's instructions (Qiagen, Germany). ITS1 sequences were amplified using primers Lig18endF (5'-GTC TTG CGG TTC ACG CTG CT-3') and Lig5.8R (5'-GAT ACT CGA GCC GAG TGA TCC-3') (Blasco-Costa et al. 2012). PCR amplifications were performed in 20 µl reactions containing 2 µl of extracted DNA, the ready-to-use 2x MyFi Mix (Bioline Ltd., United Kingdom) and 5 pmol/µl of each primer. The following thermocycling profile was applied: denaturation of DNA at 95 °C for 3 min, 35 cycles of amplification with 40 s of denaturation at 94 °C, 30 s primer annealing at 56 °C and 45 s at 72 °C for primer extension, and a final extension step of 4 min at 72 °C. PCR amplicons were purified using a Macherey–Nagel NucleoSpin® Gel and PCR Clean-Up kit (Macherey–Nagel, Germany), and PCR primers were used for sequencing. Sequencing was performed by the commercial sequence provider Macrogen (Netherlands) using ABI BigDye™ Terminator v3.1 chemistry and run on an ABI 3730XL automated sequencer. Contiguous sequences were assembled and edited using VectorNTI advance 10 (Lu and Moriyama, 2004), and the

resultant sequence identities were checked using the Basic Local Alignment Search Tool (BLAST) available from GenBank (Benson et al. 2005). The eight most complete new sequences generated in this work (GenBank accession numbers **KP294376–KP294383**) and a previously published sequence of *L. cephali* from Blasco-Costa et al. (2012) (GenBank accession number JN996865) were aligned for comparison using MUSCLE (Edgar, 2004) implemented in MEGA v5.1 (Tamura et al. 2011).

4.2.3. Geometric morphometrics

Anchor shape was analysed using landmark-based geometric morphometrics (Rohlf and Marcus, 1993; Zelditch et al. 2004; Klingenberg, 2011), which facilitates subsequent multivariate analyses (Adams et al. 2004). The anchor shape variables were obtained using eight homologous landmarks (Figure 4.1) from a sample of 213 anchors (114 ventral and 99 dorsal from 16 and 14 hosts, respectively) of 136 *L. cephali* considered as adults. The eight landmarks were chosen to represent the same biological locations and their location could be readily established in each individual (Rosenberg et al. 2002; Mitteroecker and Gunz, 2009). Landmark x and y coordinates of each anchor were obtained from digitized images with tpsDig (Rohlf, F.J., 2013, tpsDig digitise landmarks and outlines. Version 2.17. Department of Ecology and Evolution, State University of New York at Stony Brook, New York, USA) and tpsUtil (Rohlf, F.J., 2012, tpsUtility. Version 1.52. Department of Ecology and Evolution. State University of New York at Stony Brook, New York, USA) from the thin-plate spline (TPS) packages.

In order to remove all of the information unrelated to shape, the configurations were superimposed using generalised Full Procrustes Analysis (Cox and Cox, 2001; Zelditch et al. 2012; Klingenberg, 2013), using the Least Squares criterion that minimizes bending energy with respect to a mean reference form (Sarris et al. 2012). This analysis was performed with MorphoJ 1.06d (Klingenberg, 2011).

A Relative Warp Analysis (Rohlf, 1993) was performed with the Procrustes coordinates using tpsRelw (Rohlf, F.J., 2010, tpsRelw. Version 1.49. Department of Ecology and Evolution. State University of New York at Stony Brook, New York, USA) to examine shape variations in anchors among monogeneans, thereby generating a data set of shape variables. In order to give all landmarks equal weighting, the scaling option was set α= 0. The shape changes modelled onto a TPS can be separated into two parts, the uniform and non-uniform components (Rohlf and Slice, 1990). The former (U1 and U2) express global variations in shape, whereas the latter describe local shape changes at different geometric scales (Vignon et al. 2011). To visualise localised anchor shape differences, TPS deformation grids and grey-scaled coded Jacobian expansion factors, which measure the degree of local expansion or contraction of the grid (black for factors ›1, indicating expansion; grey for factors between 0 and 1, indicating contraction) were used (Bookstein, 1993; Viscosi and Cardini, 2011).

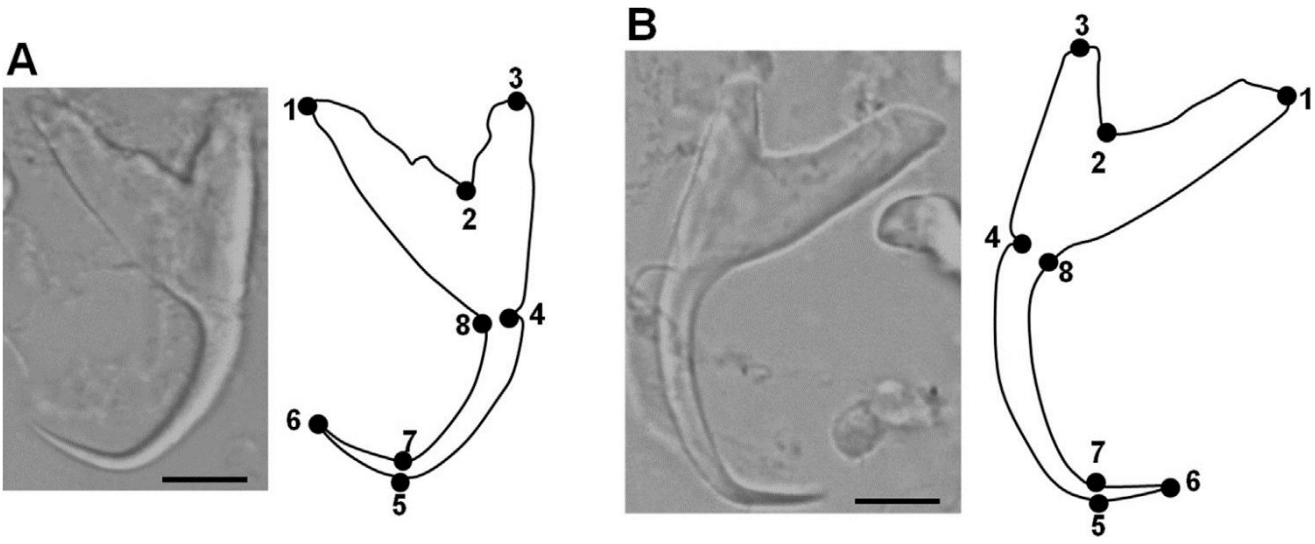

Figure 4.1. Ventral (A) and dorsal (B) anchors of *Ligophorus cephali* (micrographs and drawings). The positions of the eight landmarks were used for morphological analyses. (1) Maximum point of inner root. (2) Inflection between outer root and inner root. (3) Mean point of outer root. (4) Outer shaft base. (5) Outer point base. (6) Anchor point. (7) Inner point base. (8) Inner shaft base. Scale bar= 20 µm.

All analyses were performed separately for the two-dimensional projections of the ventral and dorsal anchor shapes. However, since we observed shape differences between the dorsal and ventral anchors, covariation in shape between them was tested in 80 specimens with matching dorsal and ventral anchors according to a two-block Partial Least Square (PLS) analysis (Rohlf, F.J., 2006, tpsPLS. Version 2.17. State University of New York at Stony Brook, New York, USA; Rohlf and Corti, 2000; Klingenberg et al. 2001). In addition, since anchors work as levers where the effort to open/close them is applied at the roots, whereas the force against the gill is applied at the point root, we also used PLS to test the covariation in shape between the root and point of dorsal and ventral anchors. For the analysis, we established two functional blocks: the "root block" (corresponding to landmarks 1–4) and the "point block" (landmarks 5–8) (Figure 4.1). The PLS analyses yielded a RV Escoufier's coefficient, which quantifies morphological integration between the blocks on a scale between 0 and 1 (the latter meaning total integration), and can be interpreted as a multivariate analogue of the coefficient of correlation (Klingenberg, 2009; Püschel, 2014).

In addition, the geometric size of each anchor was estimated as its centroid size (CS), defined as the square root of the sum of squared distances of each landmark from the centroid of the configuration (Bookstein, 1991; Zelditch et al. 2012). CS was calculated with tpsRelw 1.49 (Rohlf, 2010). Correlations analysis was used to evaluate the relationship between CS in dorsal and ventral anchors. To explore how shape variables (all relative warps) vary with CS, a multivariate regression was used for the assessment of allometric localised shape variation in ventral and dorsal anchors. In addition, the uniform component was regressed on CS to evaluate the uniform shape variation. These analyses were carried out with tpsRegr (Rohlf, F.J., 2009, tpsRegr, Shape regression. Version 1.37. Department of Ecology and Evolution. State University of New York at Stony Brook, New York, USA).

4.2.4. Data analysis with shape and size

We used Permutational Multivariate Analysis of Variance (PERMANOVA) for PRIMER (Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. Version 6. PRIMER-E: Plymouth, UK) to evaluate to what extent specific gill site variables and individual hosts accounted for shape variability in the dorsal and ventral anchors. For this purpose the gill apparatus was divided into four gill arches. Each arch was divided into four equidistant sections and three gill areas (internal, medial and external) (for details see Figure 3 in Šimková et al. (2002a, b)).

In order to estimate the components of variation in anchor shape, the relative warp datasets of dorsal and ventral anchors were used to construct respective Euclidean distance matrices. Then, we performed a PERMANOVA on the distance matrices using a crossed design with three fixed factors: gill arch (four levels), gill section (four levels) and gill area (three levels). Pseudoreplication was accounted for by considering host individual as a random factor. Due to the small sample size with respect to the number of variables and levels, our initial model included all terms up to two-way interactions. Log-transformed CS and worm size (WS), the latter measured as the area of body contours computed from digitised images, were included as covariates to control for size effects on shapes on anchors, but were tested in alternative models, and not simultaneously, to avoid the effect of collinearity (Zuur et al. 2010). The significance of each term was established based on 9999 permutations. To identify a parsimonious model of shape variation, we followed the procedure of Anderson et al. (2008). First, terms having negative and /or associated *P* values › 0.25 were pooled (one at a time and beginning with the term having the smallest mean square residual) with the term (or terms) having equivalent expected mean squares after the component of variation of the term to be pooled was set to zero. Then, the pooling of terms was repeated until all estimates of component variation associate to each term remaining in the model were positive (Anderson et al. 2008). We used a Type-I sum of squares, where each term is fitted after taking into account all

previous terms in the model. Therefore results may vary depending on the order of the terms listed in the design file (Anderson et al. 2008). However, we tried different input orders to ensure that this factor did not substantially change the resulting model (see Supplementary material 4S1).

Variation in CS was analysed as a function of the same gill-site factors considered above and host individual as random factor (for ventral anchors) with generalised Linear Mixed Models (Bates, D., Maechler, M., Bolker, B., Walker, S., 2010, lme4: Linear mixed effects models using S4 classes. Version 0.999999-0 (http://CRAN.R-project.org/package=lme4). For dorsal anchors, preliminary analyses indicated that the variance component associated with host individual was negligible and thus a Generalised Lineal Model (GLM) with the fixed factors was used instead. In addition, log-transformed WS was considered as a covariate in the models. To evaluate the influence of these explanatory variables, we first developed a series of alternative models that included different combinations of variables using a stepwise process. Model selection was based on values of the Akaike Information Criterion (AIC). Models with a difference in AIC < 2, compared with the best model, were retained (Burnham and Anderson, 2002). A model weight was computed for each of the retained models based on the value of this difference following Burnham and Anderson (2002), and a measurement of importance of each explanatory variable was obtained by summing the weights of all the models that included the given variable (Burnham and Anderson, 2002).

These analyses were performed using the lnme (Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., 2011. R Development Core Team nlme: linear and nonlinear mixed effects models. R Package Version 3, 1–102) and GLM packages in R 3.0.1 (R Development Core Team, 2011). In a preliminary analysis, the uniform components and relative warps for ventral and dorsal anchors were not significantly related to CS (r= 0.001; P= 0.990 and r= 0.004; P= 0.593, uniform components) and (r= 0.057, P= 0.545 and r= 0.047; P= 0.643, relative warps). This indicates no allometric shape variation in our dataset and allows consideration of shape and size as independent factors.

4.3. Results

4.3.1. Molecular identification

The aligned dataset of nine ITS1 sequences (eight sequences from this study and one from Blasco-Costa et al. (2012) representing *L. cephali*) was composed of 630 nucleotide (nt) positions, after trimming the end parts to match the shortest sequence. This aligned sequence set showed exactly the same pattern of nts.

4.3.2. Shape variation

A relative warp analysis was run on the total shape matrix. The first two relative warps (RW1 and RW2) accounted for 47.17% of the total variance (25.54% and 21.63%, respectively) for ventral anchors, and 45.72% (26.56% and 19.16%, respectively) for dorsal anchors. A scatter plot of RW1 and RW2, TPS and Jacobian expansion grid factors for both anchors (ventral and dorsal) are shown in Figure 4.2.

In the ventral anchors, RW1 conveyed variation in positions of the outer shaft base and inner shaft base of anchors (landmarks 4 and 8 respectively, Figure 4.2 A), defining a gradient of shaft width along this axis. In the most extreme positive values, the TPS and coded Jacobian plots indicate narrow and elongated shapes. RW2 corresponded to variation in the outer point base and inner point base (landmarks 5 and 7, respectively) of anchors, leading to different curvatures at the tips of anchors, and displayed short and wide anchors in the extreme negative values.

Regarding the dorsal anchors, the variation along RW1 mainly concerned the positions of the maximum point of the inner root, and the outer and inner point bases (landmarks 1, 5 and 7, respectively, Figure 4.2 B). Shape variation was much higher than in the ventral anchors as denoted by the TPS and Jacobian expansion grids (Figure 4.2 B). The plot indicated shortening anchor tips at the extreme positive values, similar to the ventral anchors. RW2 reflected marked variation in the maximum point of the inner root, inflection between the outer root and the inner root, the

outer shaft base, the anchor point and the inner shaft base (landmarks 1, 2, 4, 6 and 8, respectively, Figure 4.2 B). The extreme negative values showed a similar shape variation to that of ventral anchors.

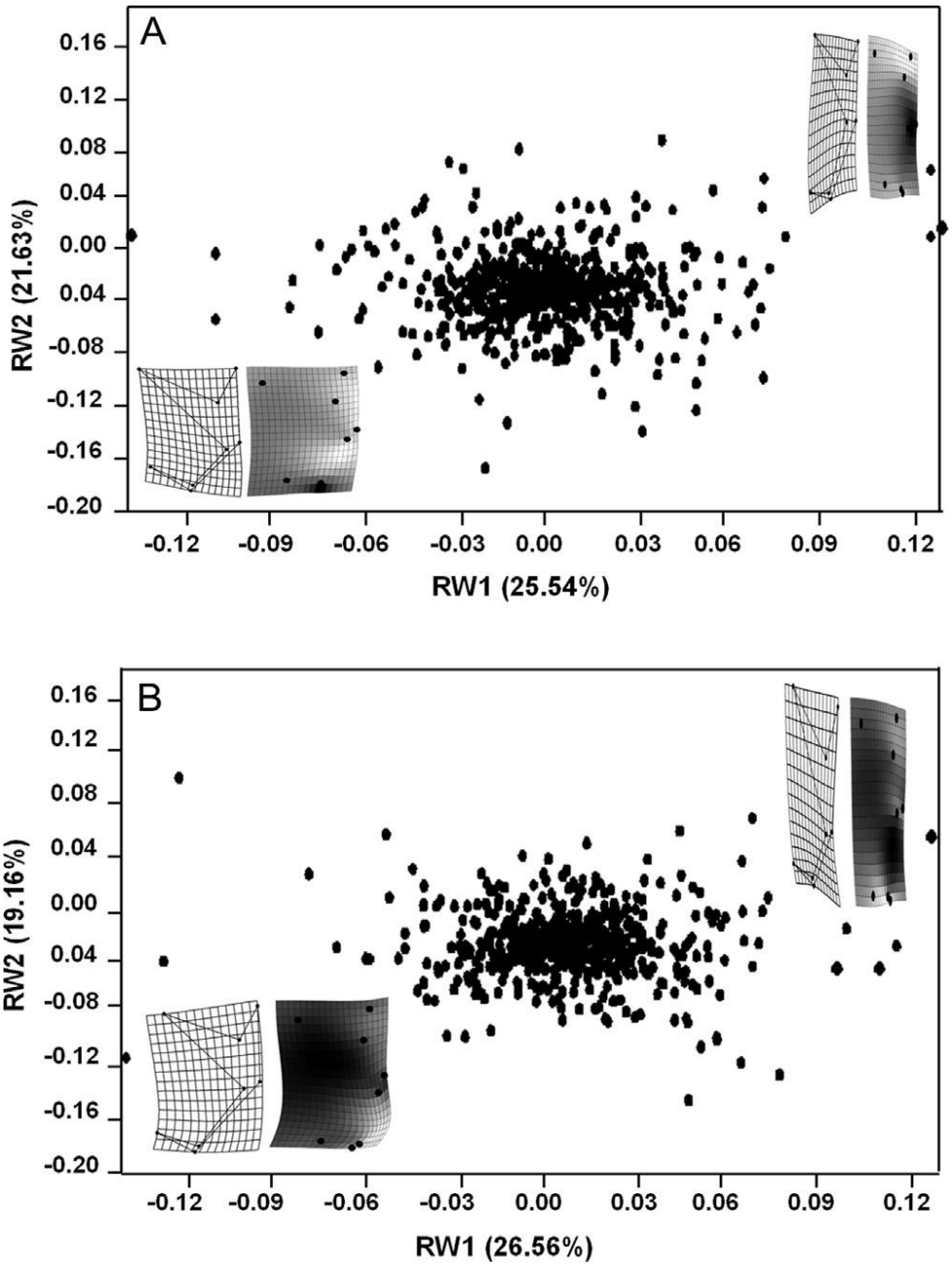

Figure 4.2. Scatterplot of relative warps 1 and 2 (RW 1 and RW 2) of the ventral (A) and dorsal (B) anchors of *Ligophorus cephali*. Points represent the positions of individual worms in the shape space. Splines associated with these first two relative warps are shown with a 2x magnification. Deformation grids indicate general shapes at the extremes of the scatterplot and grey colour coded Jacobian expansion factors convey the degree of local expansion or contraction of the grid. Values >1 indicate expansions and values between 0 and 1 indicate contraction, relative to positive and negative extremes of plot.

4.3.3. Shape and size models

Table 4.1 summarises the results of the multivariate analyses of anchor shape measurements (relative warps). Gill arch accounted for a significant part of the variation in shape of the dorsal anchors. Additionally, the variable 'gill section' explained differences in the shape of the ventral anchors, but not in a consistent manner across hosts (Table 4.1). The variation explained by model terms in the dorsal anchors was much larger (two orders of magnitude) with respect to those of the ventral anchors.

Table 4.1 Factors accounting for significant variation in the shape of ventral and dorsal anchors of *Ligophorus cephali* as revealed by a Permutational Multivariate Analysis of Variance based on pairwise Euclidean distances of relative warps coordinates.

Source of variation	Variation	*P* (perm)
Ventral anchors		
Gill section x host	$1.95\ 10^{-3}$	0.029
Residual	$7.16\ 10^{-3}$	
Dorsal anchors		
Gill arch	0.39	0.001
Residual	4.98	

P (perm), *P*-value based on random permutations.

Similarly residual variation was approximately three orders of magnitude larger in the dorsal anchors (Table 4.1).

Of the 11 candidate models considered for CS, seven were retained for the ventral anchors (Table 4.2) and three for the dorsal anchors (Table 4.3). CS of ventral anchors appeared to be mainly driven by WS and host individual, as evidenced by the inclusion of only these two variables in the

most parsimonious model (AIC: 507.1) and the estimates of relative importance (1 and 0.84 for host individual and WS, respectively), which were clearly larger than the corresponding estimates of gill area (0.35), arch (0.25) and section (0.23). The variation associated with host individual as a random factor in the best model was 3.94, which was similar to the residual variation (3.73).

Following the same criteria, gill arch was the main determinant of CS in the dorsal anchors, with a relative importance of 1 versus 0.28 and 0.21 for gill area and WS, respectively (Table 4.3). Whereas host individual accounted for a marginal part of the variation, residual variation was clearly larger than in the ventral-anchor model (15.34).

Although these results indicate high variation in the CS of ventral anchors among hosts, there was no statistical evidence that CS of either ventral or dorsal anchors was related to host size (r= 0.13; P= 0.14 and r= 0.079; P= 0.44), which is further corroborated by scatterplots showing no clear increase in CS with host weight (Figure 4.3).

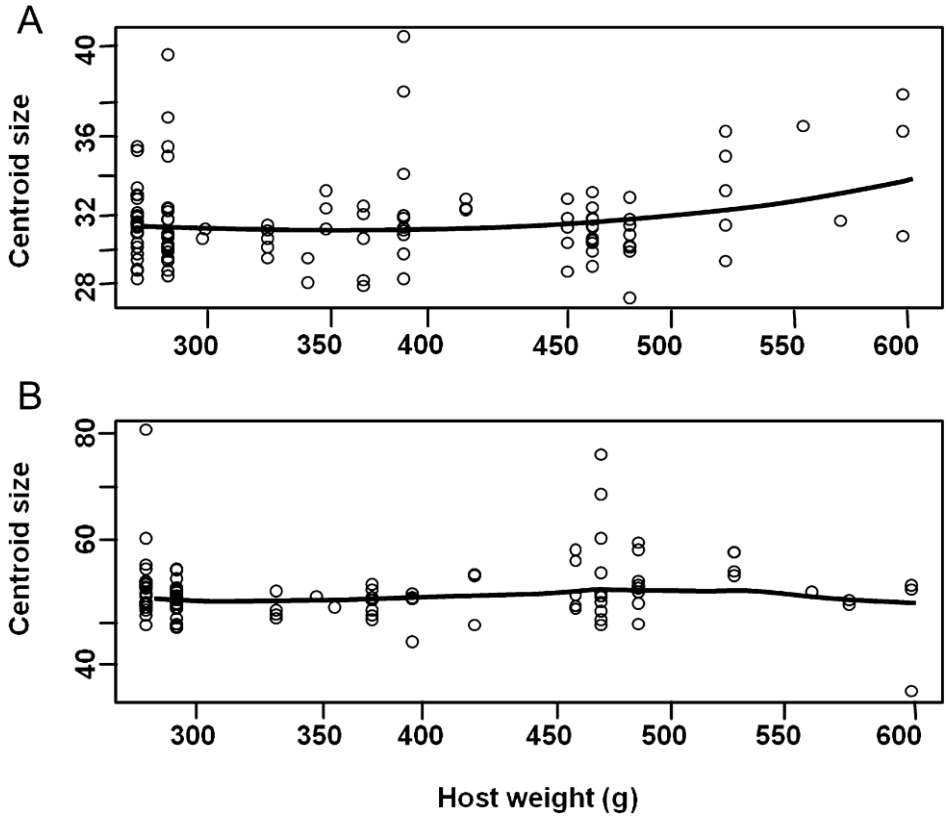

Figure 4.3. Relationship between centroid size of ventral A) and dorsal anchors B) of *Ligophorus cephali* with host weight. The trend lines are cubic smoothing splines.

Table 4.2 Generalised Lineal Mixed Models that better explain the centroid size of ventral anchors of *Ligophorus cephali* according to the values of the Akaike Information Criterion (AIC). Seven (out of 11) models with differences in Akaike Information Criterion (ΔAIC), relative to the best model, < 2 are presented (best model in bold). Weights of evidence in support of a particular model (w) are also listed.

Models	AIC	ΔAIC	*w*
CS ~ log(WS) + HOST	**507.1**	**0.0**	**0.25**
CS ~ 1 + HOST	508.0	0.9	0.16
CS ~ log(WS) + ARC + AREA + HOST	508.2	1.1	0.14
CS ~ log(WS) + SEC + HOST	508.0	1.3	0.13
CS ~ log(WS) + AREA + HOST	508.8	1.7	0.11
CS ~ log(WS) + ARC + HOST	508.8	1.7	0.11
CS ~ log(WS) + SEC + AREA + HOST	508.9	1.8	0.10

CS, centroid size; WS, worm size; ARC, gill arch; AREA, gill area; SEC, gill section; HOST, host individual (random factor).

Table 4.3 Generalised Lineal Models that better explain the centroid size of dorsal anchors of *Ligophorus cephali* according to the values of the Akaike Information Criterion (AIC) values. Three (out of seven) models with differences in Akaike Information Criterion (ΔAIC), relative to the best model, < 2 are presented (best model in bold). Weights of evidence in support of a particular model (w) are also listed.

Models	AIC	ΔAIC	*w*
CS ~ ARC	**560.2**	**0.0**	**0.50**
CS ~ ARC + AREA	561.4	1.1	0.28
CS ~ ARC + log(WS)	561.9	1.7	0.21

CS, centroid size; WS, worm size; ARC, gill arch; AREA, gill area.

4.3.4 Morphological integration

There was a slight, although significant, morphological integration between the root and point block in the same anchor (ventral anchor: *RV* coefficient= 0.40; $P \leq 0.001$; dorsal anchor: *RV*= 0.34; $P \leq 0.001$). In addition, the degree of shape integration between both the ventral anchor blocks and the dorsal anchor blocks was high (*RV*= 0.70; $P \leq 0.0001$), denoting a relatively high level of morphological integration between anchors of *L. cephali*. However, CS of ventral and dorsal anchors were not correlated ($r = -0.13$; $P= 0.18$).

4.4 Discussion

The study of sclerotized haptoral structures of monogenean morphology and phenotypic plasticity with geometric morphometrics is a poorly explored field. In the present study we believe that we use this approach for the first time to document the total morphological integration between ventral and dorsal anchors, and between the roots and points of anchors of *L. cephali*, to provide detailed information on shape variations among these anchors and to model the morphological shape and size as a function of host variables (gill arch, gill area, gill section and host individual).

The warps determine and decompose the shape variation into uniform components (global variation) and non-uniform components (local variation) (Zelditch et al. 2012). Globally, the pattern of shape variation observed herein was similar in ventral and dorsal anchors, defining a gradient ranging from narrow and elongated anchors to wide and short anchors. Similar global changes have been observed in *Gyrodactylus salaris* Malmberg, 1957 (Olstad et al. 2009). Localised shape variation in the anchors has also been reported in monogeneans of the Dactylogyridae and the Diplectanidae (Vignon and Sasal, 2010), but information is still scarce. In *Ligophorus llewellyni* Dmitrieva, Gerasev and Pron'kina, 2007, Dmitrieva et al. (2007) showed localised changes in the anchor roots and point anchors, but their study was based on linear measurements and therefore the results are not directly comparable with those of the present study.

Interestingly, localised shape variation was much higher in the dorsal anchors (compare Jacobian grids in Figure 4.2), which are in line with the higher residual variation associated with dorsal anchors in the shape models (Table 4.1). Note also that in the size models the residual variations of the dorsal anchors were much higher than those of the ventral anchors. In addition, we showed that random effects (gill section x host individual) were an important determinant of shape in ventral, but not in dorsal, anchors and size models of dorsal and ventral anchors were clearly different (Tables 4.2 and 4.3).

All of this evidence points to differences between dorsal and ventral anchors in the factors determining both shape and size, which perhaps reflects different functional roles in attachment to the gills. To our knowledge, detailed functional studies of the hard haptoral structures in *Ligophorus* are lacking and it is therefore difficult to interpret our results in the light of current evidence. However, in *Ligophorus* the pairs of ventral anchors and dorsal anchors are connected, respectively, by ventral and dorsal transverse bars. In *L. cephali*, as in other species of the genus, these bars are dissimilar in shape and size (Siquier and Ostrowski de Núñez, 2009; Sarabeev et al. 2013) and the ventral bar appears to be more rigid than the dorsal one (Dmitrieva et al. 2012). In fact, the curvature of the dorsal bar can vary sharply (Mariniello et al. 2004; Dmitrieva et al. 2007; Sarabeev et al. 2013). Thus morphology suggests that the dorsal anchor/bar complex is more mobile than the ventral one, at least in this genus.

Arya and Singh (2013) observed the movement and change in position/orientation of various haptoral elements with respect to the dorsal anchors in *Mizelleus indicus* (Jain, 1957) (Dactylogyridae) from *Wallago attu* (Bl. and Schn.). Although the morphology of the bars and anchors in this species is quite different from that of *Ligophorus* spp., some of their findings appear useful in understanding some aspects on the functional dynamics of anchors and bars of *L. cephali*. In *M. indicus* the process of achieving attachment to the host tissue involves movements of the ventral bar together with the ventral anchors. This movement is achieved with or without the aid of the

supporting dorsal bar, which moves upwards and downwards, resulting in spreading the points of the dorsal anchors. Thus the dorsal bar appears to be primarily involved in the movement of the dorsal anchors.

In light of this evidence, the differences in forces generated for attachment by the respective bars might account for the differences between the dorsal and ventral anchors observed in the present study. The lower residual variation associated with the ventral anchors suggests a tighter control of their shape and size, perhaps because these anchors are the most important for attachment and their size and shape would more closely fit the characteristics of the individual host microenvironment (Šimková et al. 2001; Mancheva et al. 2009; Sarabeev et al. 2013). This is also in line with the significant fraction of variation accounted for by host-associated random effects in the ventral anchors in the models of anchor shape and size.

Despite these putative functional differences, we observed high integration in shape between the ventral and dorsal anchors, indicating a concerted action between dorsal and ventral structures. Vignon et al. (2011) also suggested strong coordination and integration among the different parts of the haptoral structure in *Cichlidogyrus* spp., (Monogenea, Dactylogyridae) considering three main morphological configurations in the parts of attachment organs as modules: marginal hooks, anchors and bars. Thus, their results revealed that the shapes of haptoral parts are not independent characters and furthermore suggest morphological integration, which is in line with our findings.

This coordination among parts of the haptor could be due to host specificity and the attachment mechanism (Vignon et al. 2011). Klingenberg (2008) suggested that this kind of morphological covariation can play a substantial role in determining the evolutionary potential of traits within populations. Although the haptoral structures have long been studied in various environmental and evolutionary contexts, our study highlights the importance of morphological integration analyses for better understanding of the variability among haptoral anchors.

A considerable part of the variation in the shape and size models was attributable to either random factors or remained unaccounted for by the variables considered. Thus there is a large unpredictable component in the models imputable to a combination of measurement error, genetic variation, ontogenetic changes and plastic responses to environmental factors. The molecular analyses showed that the ITS1 sequences of our specimens were identical to those of *L. cephali* previously reported in a nearby locality (Cullera) by Blasco-Costa et al. (2012). ITS1 sequences have previously shown some level of intraspecific divergence within species of monogeneans, including members of *Gyrodactylus* (0.09–3.5% intraspecific divergence, Bueno-Silva et al. 2011), *Lamellodiscus* (0.27%, Desdevises et al. 2000) and *Furnestinia* (0.05–1.38%, Mladineo et al. 2013). This evidence does not completely rule out some level of genetic variation in our sample and it is therefore possible that not all of the phenotypic variation revealed in the present investigation is environmentally induced. However, we ensured that all of the specimens of *L. cephali* used in the present study came from fish captured within 1 day in a single locality in L'Albufera (El Palmar), thereby reducing the possibility of important genetic differences. Note also that anchor shape was independent of WS and therefore ontogenetic changes do not seem to contribute substantially to anchor shape. This lack of relationship with WS was also observed by Dmitrieva and Dimitrov (2002) in haptoral structures in gyrodactylids. They observed that the size of the anchors is the most variable, whereas the size of the marginal hooks is the most stable. This is associated with the order of appearance of these structures in ontogeny. Marginal hooks, which appear first, can reach their final size long before the birth, whereas the size of the anchors, which appear later, is essentially dependent on the duration of embryogenesis. It is therefore likely that much of the random variation reported herein reflects environmentally driven plastic responses.

In addition, we found that host gill arch was an important determinant of anchor shape and size in the dorsal anchors. Shape variability related to the host gill arch has also been observed in *L. imitans* and others monogeneans (Caltran et al. 1995b; Roberts and Janovy, 1996). This is perhaps

not surprising given that hydrodynamic processes are associated with the spatial position of each gill and this can determine the leverage applied for attachment (Soler-Jiménez and Fajer-Ávila, 2012). In fact, maintaining high phenotypic plasticity can be advantageous in monogeneans given the diversity of microhabitats provided by fish gills (Šimková et al. 2002a, b; Šimková et al. 2004; Verneau et al. 2009) and thus selective forces can promote the maintenance of this feature.

Phenotypic plasticity allows organisms to respond rapidly to changing environmental conditions without the time lag required for responses to natural selection (Zhou et al. 2012). The evidence presented herein points to phenotypic plasticity in anchor morphology, which could confer on *Ligophorus* spp. the ability to instantly colonise a new host when the occasion arises. In fact, straggling seems common in this genus due to the usual co-occurrence of several sympatric host populations that overlap in habitat and behaviour, and which, due to their phylogenetic relatedness, can provide a similar physiological environment for the parasites (Sarabeev et al. 2013; Sarabeev and Desdevises, 2014). Eventually straggling would make host switching and subsequent speciation in the newly colonised host possible, as postulated by Desdevises (2007).

We found no evidence of correlation between dorsal or ventral anchors size and host size. This relationship has been much studied in monogeneans, and most evidence points to a significant positive correlation between these traits (Perera, 1992; Šimková et al. 2006; Mendlová and Šimková, 2014), including species of *Ligophorus* (Caltran et al. 1995b; Rubtsova et al. 2005). This pattern has often been explained in terms of water currents and the secondary lamella lengths that tend to increase with host gill size and the performance of the parasite's attachment to the host gill that is associated with parasite anchor size (Kearn, 1970; Caltran et al. 1995b; Turgut et al. 2006; Soler-Jiménez and Fajer-Ávila, 2012). However, other studies do not support this relationship (Fuentes and Nasir, 1990; Matejusová et al. 2002). In *Metamicrocotyla macracantha* Alexander, 1954 from *M. cephalus*, the unique perpendicular attachment of the parasite haptor to the host gill filament seems

to limit the ability of the haptor to grow past the maximum width of the host gill filament, even while the body of the worm continues to grow relative to the haptor (Baker et al. 2005).

However, it seems unlikely that this type of constraint affects *L. cephali*. Note, however, that the range of host sizes in the present study is quite narrow, which could determine the lack of relationship with anchor size. In addition, previous studies based on linear measurement did not explicitly separate size and shape. Therefore this question deserves further exploration within a geometric morphometric framework.

We observed shape integration within parasite anchors (point and root blocks), which is not surprising given the functional relationship between points and roots. However, in line with previous findings in *L. imitans*, Caltran et al. (1995a) reported that, in the same anchor, not all of the metric variables are systematically positively correlated, which the authors interpreted as resulting from asynchronous growth of the different anchor parts. In any case, the low integration observed herein indicates that a large fraction of the phenotypic variation observed does not compromise the functional role of anchors as levers.

The present study demonstrates that geometric morphometrics can be an extremely useful technique in analysing intraspecific shape and size variations in haptoral structures in monogeneans and illustrates the potential to provide new insight into the functional morphology of parasite attachment to the host and evolutionary processes of host-parasite coevolution. Additionally, future studies should assess the patterns of shape evolution in the genus, assessing the quantitative genetics of shape variation.

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Supplementary material

Supplementary Data 4S1. Design models used in the Permanova analysis for ventral and dorsal anchors. All factors evaluated were fixed factors, except the host which was taken as random factor.

Ventral anchors

Design 1: Gill arch – Gill section – Gill area – Host

Gill arch

Gill section

Gill area

Host

Design 2: Gill section – Gill area – Host – Gill arch

Gill section

Gill area

Host

Gill area

Design 3: Gill area – Host – Gill arch – Gill section

Gill area

Host

Gill arch

Gill section

Design 4: Host – Gill arch – Gill section – Gill area

Host

Gill arch

Gill section

Gill area

Design 5: Gill section – Host – Gill arch

Gill section

Host

Gill arch

Design 6: Gill section – Host – Gill area

Gill section

Host

Gill area

Design 7: Host – Gill section – Gill area

Host

Gill section

Gill area

Dorsal anchors

Design 1: Gill arch – Gill section – Gill area – Host

Gill arch

Gill section

Gill area

Host

Design 2: Gill section – Gill area – Host – Gill arch

Gill section

Gill area

Host

Gill arch

Design 3: Gill area – Host – Gill arch – Gill section

Gill area

Host

Gill arch

Gill section

Design 4: Host – Gill arch – Gill section – Gill area

Host

Gill arch

Gill section

Gill area

Design 5: Host – Gill arch – Gill section

Host

Gill arch

Gill section

Design 6: Gill arch – Gill section – Host

Gill arch

Gill section

Host

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CHAPTER 5

A New Species of *Ligophorus* (Monogenea: Dactylogyridae) from the gills of the Flathead Mullet *Mugil cephalus* (Teleostei: Mugilidae) from Mexico

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Abstract

A new monogenean species, *Ligophorus yucatanensis* n. sp. from the gills of the flathead mullet *Mugil cephalus* from the Yucatan Peninsula, Mexico, is described. The new species can be differentiated from all other species of *Ligophorus* by the morphology of the accessory piece of the copulatory organ. Its main lobe is cylindrical, tunnelled expanded distally, slightly bowed with a characteristic membranous opening at level of medial bifurcation of the accessory piece, forming a thick-walled bulb shaped expansion that ends in a round labium. The secondary lobe is spatulate, straight, and shorter than the main lobe. In addition, the new species can be distinguished from other species by the morphology of the haptoral ventral bar, and the distal end of the vaginal duct. Furthermore the ventral anchors are shorter than those of all other species of *Ligophorus* reported in the Gulf of Mexico and Caribbean Sea. In addition, the zoogeographical records of *Ligophorus* spp. on the *M. cephalus* species complex are briefly reviewed and updated.

Keywords: Monogenea, *Ligophorus*, Flathead mullet, Yucatan Peninsula, Gulf of Mexico.

5.1. Introduction

Ligophorus Euzet and Suriano, 1977 includes 60 valid species of monogeneans infecting the gills of grey mullets (Mugilidae) (Dmitrieva et al. 2012; Soo and Lim, 2012, 2015, El Hafidi et al. 2013a, b; Kritsky et al. 2013; Sarabeev et al. 2013; Soo et al. 2015). Species of the genus are characterized by the combination of the following features: vas deferens on the left side not encircling the intestinal caeca; one prostatic reservoir; copulatory complex comprising a copulatory organ with bilobed base and accessory piece; a J – to U-shaped ovary, a vagina sclerotized or not; dorsal and ventral anchor/ bar complex with seven pairs of hooks and bars dissimilar in shape, ventral bar with anteromedian protuberances (Sarabeev et al. 2013).

Ligophorus spp. are restricted to species of Mugilidae worldwide. A sizeable proportion of species (19) have been recorded on the cosmopolitan flathead mullet *Mugil cephalus* Linnaeus 1758 (Sarabeev et al. 2013). However, recent evidence indicates that this host denomination probably represents a complex of about 14 species (including also *M. liza* Valenciennes, 1836 and *M. platanus* Günther 1880), each with a different regional distribution (Shen et al. 2011; Durand et al. 2012; Whitfield et al. 2012). Given that evidence from host-parasite records suggests that the species of *Ligophorus* are fairly oixenic (Sarabeev et al. 2013), it has been proposed that those occurring on *M. cephalus* sensu lato may serve as host markers at regional scale (El Hafidi et al. 2013a).

To date, only a record of *Ligophorus* from *M. cephalus* has been reported from the Gulf of Mexico, *Ligophorus mugilinus* (Hargis, 1955) Euzet and Suriano 1977 (Sarabeev et al. 2005). *L. mugilinus* has also been reported in the Caribbean on *M. curema* Valenciennes 1836 (Fuentes and Nasir, 1990; Bunkley-Williams and Williams, 1994), but the specific ascription of these forms is uncertain (Sarabeev et al. 2005). We have recently collected specimens of *Ligophorus* from *M. cephalus* in coastal waters of Yucatan, Mexico, and the morphological study of this material suggests that they represent a new species of *Ligophorus*, which is described herein. The present description is based chiefly on the

morphology of the sclerotized parts because the condition of the specimens precluded a detailed study of their soft anatomy. However, the taxonomy of the genus relies mostly on the morphology of the male copulatory complex, vaginal duct and haptoral structures and, therefore, the morphological evidence provided herein is sufficient to justify the erection of a new species (Sarabeev et al. 2005; Sarabeev et al. 2013). Additionally, the present study briefly updates the zoogeography of *Ligophorus* spp. on *M. cephalus* sensu lato, in the light of current evidence for a complex of cryptic species (Durand et al. 2012; Whitfield et al. 2012).

5.2. Materials and Methods

Flathead mullets were caught using hook and line and throw nets in Celestun Lagoon (Figure 5.1) at the northwest of the Yucatan Peninsula, Mexico. Thirteen specimens of *Mugil cephalus* were caught within a comprehensive fish survey during July 2011 and May 2012. Flathead mullets were kept on ice for about 24 hours prior to freezing upon return to the laboratory. Their total length (Mean ± SD: 33.4 ± 7.8 cm) and weight (495 ± 386 g) were recorded.

The gills of each host were removed and examined under a dissection microscope and the monogeneans obtained were preserved in 96° alcohol, labeled and stored in vials for later evaluation. The monogeneans were treated using a mixture of 300 µl of TE9 buffer (500 mM Tris-HCl, 200 mM EDTA, 10 mM NaCl, pH 9) and 100–200 µl of proteinase K (10 mg/ml) (Modified of Mo and Appleby 1990; Paladini et al. 2011) to digest the soft tissues, and then the specimens were mounted on microscope slides with glycerogelatin (Sarabeev et al. 2005).

We measured 28 metric characters as defined by Sarabeev et al. (2013): VAA, ventral anchor inner length; VAB, ventral anchor main part length; VAC, ventral anchor outer root length; VAD, ventral anchor inner root length; VAE, ventral anchor point length; VAF, ventral anchor shaft length; BL, blade of anchor; VAG, ventral anchor outer length; DAA, dorsal anchor inner length; DAB, dorsal anchor main part length; DAC, dorsal anchor outer root length; DAD, dorsal anchor

inner root length; DAE, dorsal anchor point length; DAF, dorsal anchor shaft length; DAG, dorsal anchor outer length; HTL, hook total length; VBL, ventral bar length; VBDP, distance between anterior protuberances of ventral bar; DBL, dorsal bar length; APTL, copulatory complex accessory piece total length; APML, copulatory complex accessory piece main lobe length; APMW, copulatory complex accessory piece distal portion of main lobe width; APSL, copulatory complex accessory piece secondary lobe length, COL, total length of copulatory organ; COW, copulatory organ width at midlength; VL, vagina length.

Drawings and measurements were made with the aid of a drawing tube using an Nikon (Tokyo, Japan) Optihot-2 microscope with interference contrast, and photographs were made with a Leica (St. Gallen, Switzerland) DMR microscope with interference contrast and a Leica DFC295 camera.

Average measurements (all in µm) and standard deviation are followed by ranges in parentheses and the number of observations and structures measured as N. The drawings were made using a 100x objective for sclerotized structures. The term "prevalence" is used herein following Bush et al. (1997). Type and paratypes specimens were deposited in the Natural History Museum, London (NHMUK).

***Ligophorus yucatanensis* n. sp.** (Figures. 5.2, 5.3 A-H)

Type-host: Flathead mullet, *Mugil cephalus* Linnaeus, 1758.

Type-locality: Celestun Lagoon, State of Yucatan, Mexico (20° 52'N, 90° 22' W).

Type-material: Holotype and 2 paratypes deposited at the NHMUK (registration numbers 2014.5.1.1., and 2014.5.1.2-3, respectively).

Site of infection: Gills.

Prevalence of infection: 11 of 13 *M. cephalus* examined (77%).

Etymology: The specific designation *yucatanensis* refers to the collection site.

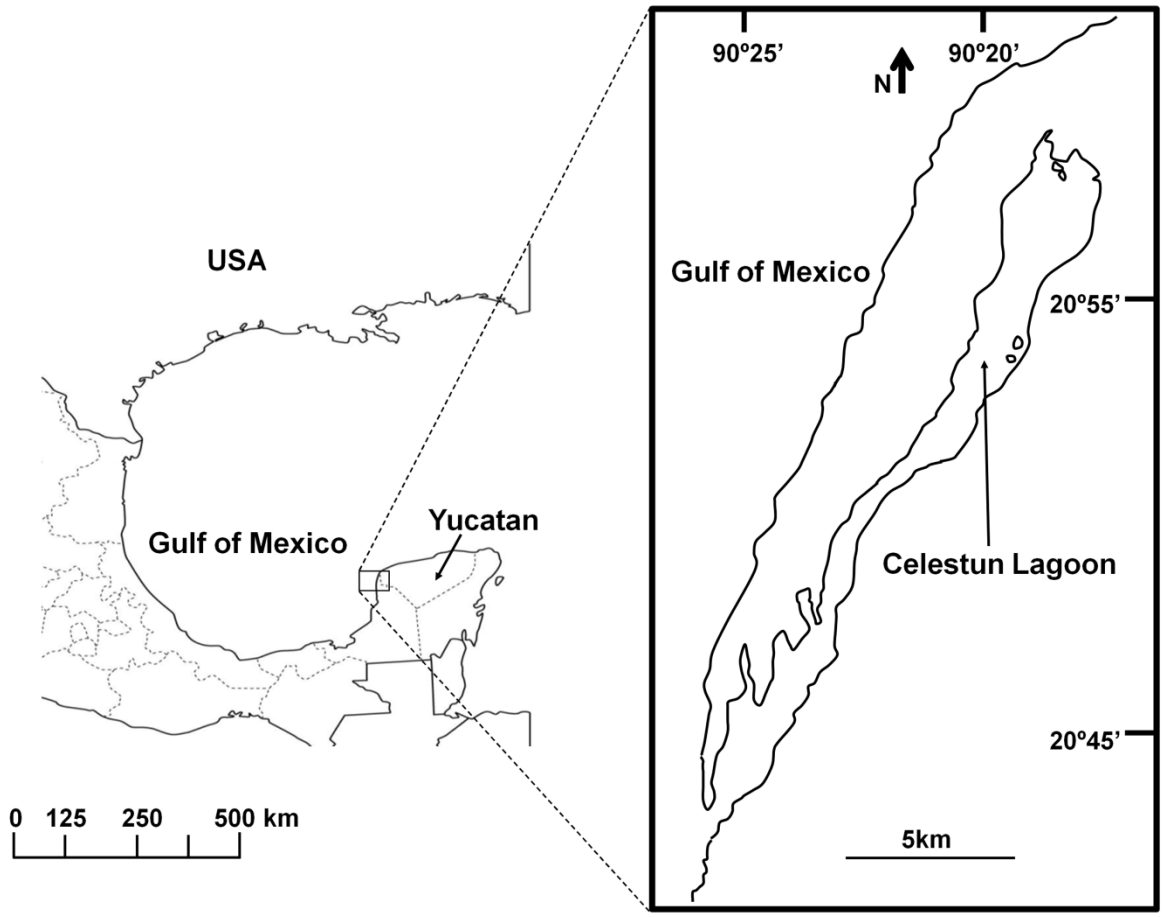

Figure 5.1. Location of Celestun Lagoon, type locality of *Ligophorus yucatanensis* n. sp.

5.2.1. Description

Morphometric measurements based on 10 specimens presented in Table 5.1. Worms with characters of genus as defined by Euzet and Suriano (1977), and supplemented by Sarabeev and Balbuena (2004) and Sarabeev et al. (2013). Haptor armed with 7 pairs of marginal hooks, four anchors and two transverse bars (Figure 5.3B). Body elongated. Dorsal and ventral anchors with elongate thin blade, and recurved point (Figures 5.3C, D). Pairs of anchors differ more in size than in shape (Figure 5.3B), inner length of ventral anchor less or equal than that of dorsal anchor. Both anchors with sharply bent blade, shaft about 1.5 to 2.3 times longer than point, the latter not reaching level of tip of inner root, outer root shorter and more slender than inner root; outer root and point subequal in length.

Ventral anchors connected by transverse ventral bar (Figures 5.3G, H). Bars subequal in length; finger-like anterior protuberances of ventral bar situated ventrally, not reaching level of dorsal side of bar; ventral knot absent; anteromedian process present, small, knoll-shaped; the median groove shallow and lateral flaps (Figure 5.3G). Transverse dorsal bar yoke-shaped, with enlarged and rounded extremities, connects dorsal anchors (Figure 5.3H); posteromedian process poorly developed or absent. All 14 marginal hooks subequal in shape and size (Figure 5.3E), with short base, heel, curved blade and filament loop. Handle straight, slightly inflated at distal end.

Male copulatory organ C-shaped (Figures 5.2, 5.3A), long, thin, enters accessory piece distally; heel of base of copulatory organ developed and bulb of copulatory organ base thick-walled (Figure 5.2); accessory piece claw-shaped, pincer-like; secondary lobe joins main lobe, articulated medially by simple joint. Main lobe of accessory piece cylindrical, tunnelled expanded distally, slightly bowed with characteristic membranous opening at level of medial bifurcation of accessory piece forming thickwalled bulb-shaped expansion terminating in round "labium"; secondary lobe spatulate, straight, shorter than main lobe, not reaching level of tip of distal end of main lobe.

Bulb of copulatory organ base bifurcates into 2 terminal lobes, similar in width and distinct in shape. Vaginal duct short, distal end scyphoid, narrow; consists of a bulb that turns into typically inconspicuous extinction; aperture midventral (Figures 5.2, 5.3F).

5.2.2. Remarks

Given the high host specificity of the species of *Ligophorus*, (Sarabeev and Desdevises, 2014) the present comparison of *L. yucatanensis* n. sp. focuses mostly on species described on *M. cephalus* sensu lato. Geographically the closest congeneric species to *L. yucatanensis* n. sp. is *L. mugilinus* originally recorded in the Gulf of Mexico (Hargis, 1995). *L. yucatanensis* n. sp. is similar to this species, as redescribed by Sarabeev et al. (2013), with respect to the distal end of the vagina, which is scyphoid

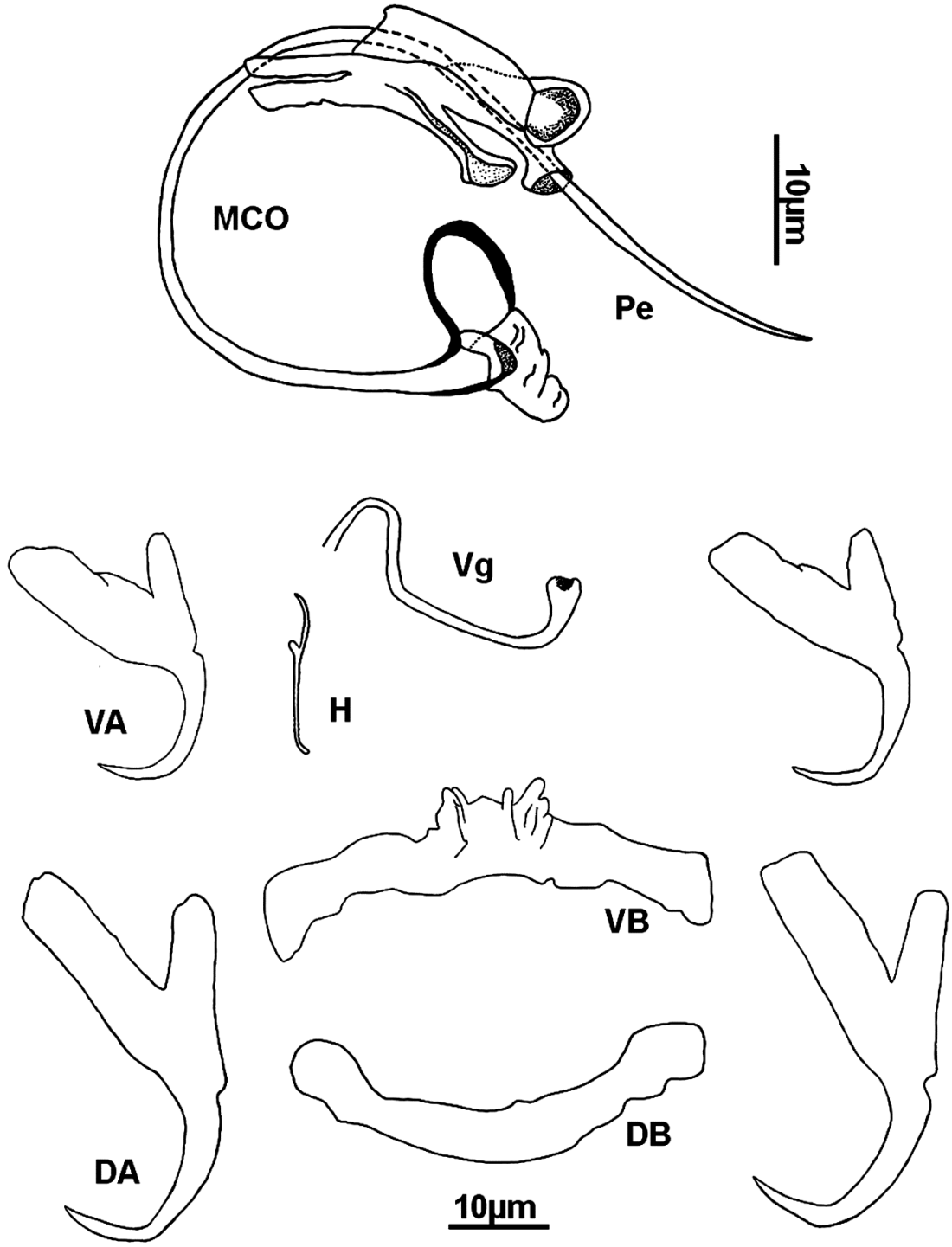

Figure 5.2. Haptoral and copulatory hard parts of *Ligophorus yucatanensis* n. sp. from *Mugil cephalus*. Abbreviations: VA. ventral anchor; VB. ventral bar; DA. dorsal anchor; DB. dorsal bar; H. hook; MCO. male copulatory organ; Pe. penis; Vg. vagina. Scale bar= 10 µm.

and narrow, and the shape of the ventral and dorsal anchors, exhibiting a sharply bent blade in both species.

However, *L. yucatanensis* n. sp. differs markedly from *L. mugilinus* in the morphology of the accessory piece, which is cross-shaped in the latter, whereas it is claw-shaped in *L. yucatanensis* n. sp. The main lobe and secondary lobe of the accessory piece of the male copulatory complex are also clearly different; in *L. mugilinus* the main lobe is slightly bowed, elongated, cylindrical and the secondary lobe is massive, V-shaped with unequal branches, extending beyond the distal end of the main lobe. By contrast, in *L. yucatanensis* n. sp. the main lobe is tunnelled, expanding distally, and exhibits a thick-walled bulb-shaped opening. In addition, the secondary lobe is massive V-shaped in *L. mugilinus* vs. spatulate in *L. yucatanensis* n. sp. The new species also differs from *L. mugilinus* in the shape of the ventral bar. In the former it has finger-like anterior protuberances on its ventral side and the anteromedian process is knoll-shaped on the ventral shield; whereas in *L. mugilinus* an ∩-shaped knot of the ventral shield is attached to a Λ-shaped dorsal anteromedian process (Sarabeev et al. 2013).

L. yucatanensis n. sp. resembles *Ligophorus chabaudi* Euzet et Suriano 1977, *Ligophorus cephali* Rubtsova, Balbuena, Sarabeev, Blasco-Costa et Euzet, 2006 and *Ligophorus mediterraneus* Sarabeev, Balbuena et Euzet, 2005 occurring on *M. cephalus* in the Mediterranean Sea by possessing a C-shaped, long and thin copulatory organ; where the base of the latter has a thick-walled heel. In addition, the ventral and dorsal anchors of the four species are similar in shape and size; with a sharply bent blade. Furthermore, in *L. chabaudi*, *L. cephali* and in the new species the accessory piece is claw-shaped, pincer-like and the distal end of the copulatory organ enters the accessory piece distally.

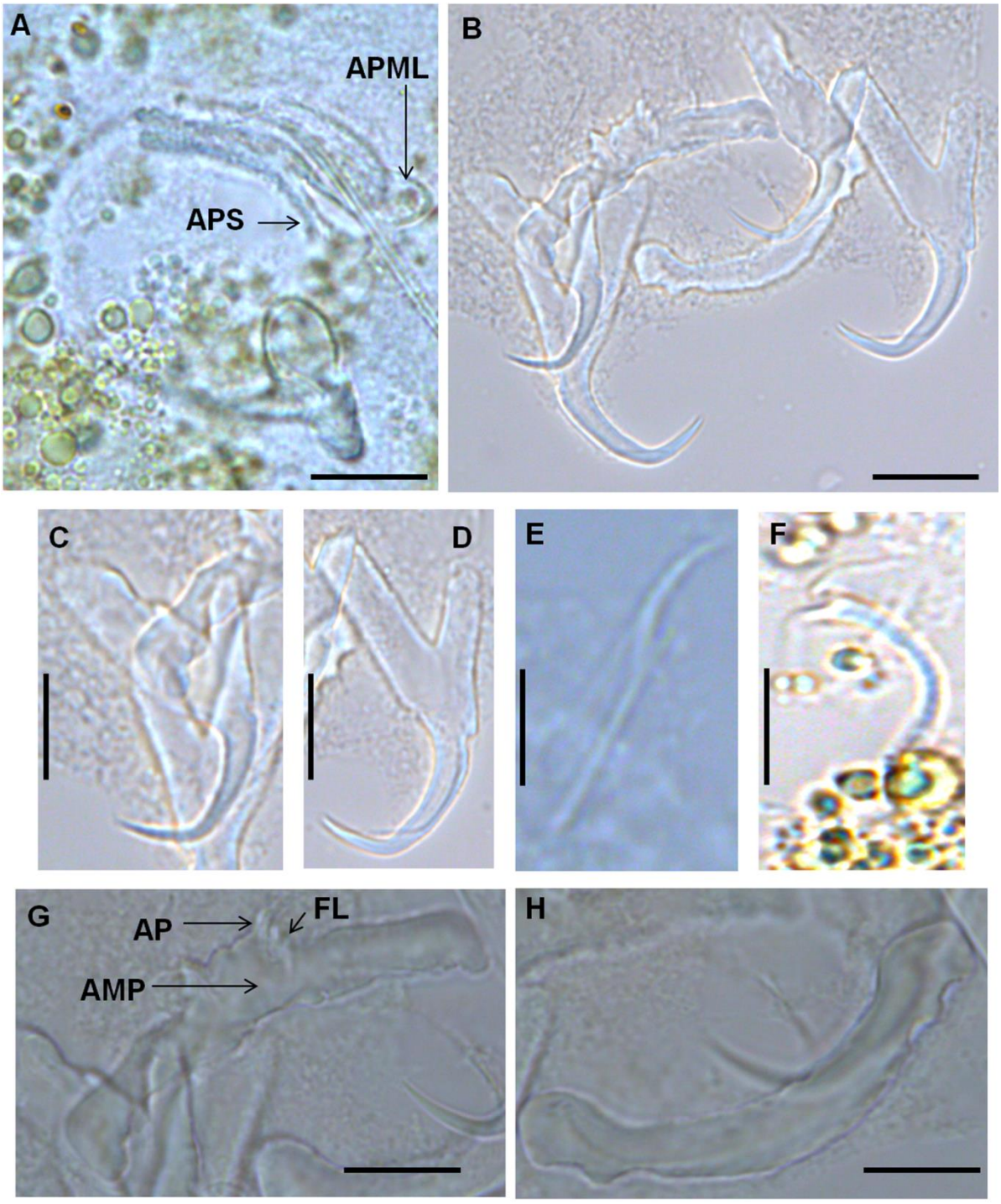

Figure 5.3. Interference contrast microscope images of haptoral and copulatory hard parts of *Ligophorus yucatanensis* n. sp. Abbreviations: A. male copulatory organ; B. haptor; APML. copulatory complex accessory piece main lobe length, APS. accessory piece of copulatory complex secondary lobe, C. ventral anchor; D. dorsal anchor; E. marginal hook; F. vagina; G. ventral bar; AP. anterior protuberances of ventral bar; AMP. anteromedian process of ventral bar; FL. Flaps of shield of ventral bar; H. dorsal bar. Scale bars: A, E, F, G, H= 10 µm; B, C, D=20 µm.

However, *L. yucatanensis* n. sp. differs from *L. chabaudi* in the morphology of the main lobe of the accessory piece. It is straight in the latter, whereas in *L. yucatanensis* n. sp. shows a membranous opening at the level of the medial bifurcation of the accessory piece. The ventral bar of both species is also different. In *L. yucatanensis* n. sp. it has finger-like anterior protuberances situated ventrally, not reaching the level of the dorsal side of the bar and the anteromedian process is small, knoll-shaped, whereas in *L. chabaudi* the dorsal anteromedian process is absent and the finger-like anterior protuberances extend beyond the level of the dorsal side of the bar and form a conspicuous V-shaped structure. Furthermore, the distal end of the vagina is scyphoid in *L. yucatanensis* n. sp. vs. funnel shaped in *L. chabaudi*.

L. yucatanensis n. sp. can also be readily distinguished from *L. cephali* in the morphology of the secondary lobe of the accessory piece, being spatulate, straight, short in the new species vs. winding, tubular and long in *L. cephali*. In addition, the ventral bar in *L. cephali* has a Λ-shaped dorsal anteromedian process, which is absent in *L. yucatanensis* n. sp. and the distal end of the vaginal duct is scyphoid in *L. yucatanensis* n. sp. vs. funnel-shaped in *L. cephali*.

L. mediterraneus differs markedly from *L. yucatanensis* n. sp. in the accessory piece of the copulatory organ, which is cross-shaped vs. claw-shaped in *L. yucatanensis* n. sp. and the distal end of the copulatory organ enters the accessory piece proximally in *L. mediterraneus* vs. distally in the new species. In addition, the secondary lobes of the accessory piece are markedly distinct, being spatulate, straight and short in *L. yucatanensis* n. sp. vs. massive, V-shaped in *L. mediterraneus*.

L. yucatanensis n. sp. can also be distinguished by the morphology of the ventral bar, which lacks a knot on the ventral shield, whereas in *L. mediterraneus* it is present and V-shaped.

L. yucatanensis n. sp. also resembles *Ligophorus maroccanus* El Hafidi, Berrada Rkhami et Pariselle 2013a occurring on *M. cephalus* off Western Sahara in the shape of ventral anchors with shaft shorter than guard. The new species differs in the morphology of the ventral bar being V-shaped in *L.*

maroccanus vs. knoll-shaped in *L. yucatanensis* n. sp., with two anterior protuberances of ventral bar for both species; Also differs clearly in the morphology of the vagina that is tubular with sclerotized walls in *L. maroccanus* and scyphoid in *L. yucatanensis* n. sp. and in the accessory piece of the male copulatory organ: In *L. maroccanus* accessory piece is bifurcated and substantially longer than in *L. yucatanensis* n. sp.

With respect to species from Sea of Japan and Yellow Sea commonly occurring on *M. cephalus*, *L. yucatanensis* n. sp. resemble *Ligophorus abditus* Dmitrieva, Gerasev et Gibson, 2013, *Ligophorus pacificus* Rubtsova, Balbuena et Sarabeev, 2007, *Ligophorus domnichi* Rubtsova, Balbuena et Sarabeev, 2007, *Ligophorus cheleus* Rubtsova, Balbuena et Sarabeev, 2007, *Ligophorus chenzhenensis* Hu et Li 1992 in the morphology of the copulatory organ, which is C-shaped, long and thin, entering the accessory piece distally and exhibiting a thick-walled heel at its base,; the accessory piece is claw claw shaped, pincerlike, its main lobe is cylindrical, expanded distally, slightly bowed, and in the morphology of the anchors with sharply bent blade.

However, *L. yucatanensis* n. sp. differs from *L. abditus* in the distal part of main lobe exhibiting a round "labium" in the new species and being beak-shaped in *L. abditus*. Both species also differ in the morphology of the ventral bar with small, knoll-shaped anteromedian process in *L. yucatanensis* n. sp. vs. knot present in *L. abditus*. The distal ends of the vagina is also different, being scyphoid in *L. yucatanensis* n. sp. vs. funnel-shaped in *L. abditus*. *L. yucatanensis* n. sp. also differs with *L. pacificus* in the morphology of ventral bar, which possess a V-shaped knot on the ventral shield in the latter, whereas the ventral knot is absent in the new species, and the anteromedian process is absent in *L. pacificus* and knoll-shaped in the new species. Finally, the distal part of the accessory piece of the copulatory organ is membranous and subtriangular in *L. pacificus* vs. tunnelled expanded distally in *L. yucatanensis* n. sp.

Likewise, *L. yucatanensis* n. sp. differs from *L. domnichi* in the morphology of the ventral bar, which has a V- or T-shaped knot on the ventral shield in the latter, whereas the knot is absent and anteromedian knoll-shaped process is present in *L. yucatanensis* n. sp. In addition, the distal part of the accessory piece is heavily-sclerotized and shows a bulb shaped expansion in *L. domnichi*, whereas it is cylindrical, tunnelled and expanded distally in *L. yucatanensis* n. sp. In addition, anteromedian process present in both species, and anterior protuberances reduced dorsally in *L. domnichi* and ventrally not reaching level of dorsal side of bar in *L. yucatanensis* n. sp.

L. yucatanensis n. sp. also differs in the morphology of ventral bar with *L. cheleus*. The anteromedian process is absent and the knot is V-shaped in the latter, whereas the anteromedian process is knoll-shaped and the knot is absent in *L. yucatanensis* n. sp. In addition, the morphology of the accessory piece of the copulatory organ is very different in both species. In *L. yucatanensis* n. sp. the secondary lobe of the accessory piece shows an expansion distally vs. not expanded in *L. cheleus* and the medial expansion of the accessory piece is bulb-shaped in the former vs. straight in the later.

Likewise, *L. yucatanensis* n. sp. and *L. chenzhenensis* clearly differs in the main lobe of accessory piece expanded distally and tunnelled in the new species, and bifurcated in *L. chenzhenensis*. In this species, the main lobe is straight with a sharply curved distal end whereas it is tunnelled and expanded distally in the new species. In addition the anteromedian process of the ventral bar is absent or poorly developed in the latter vs. present and knoll-shaped in *L. yucatanensis* n. sp.

In addition, *L. yucatanensis* n. sp. resemble *Ligophorus huitrempe* Fernández 1987 from the Southeast Pacific in the shape of the copulatory organ, which is C-shaped, long, thin with a thick-walled heel at its base and the distal end of the vagina is scyphoid and narrow. However, both species differ markedly in the accessory piece, being cross-shaped in *L. huitrempe* vs. claw-shaped in *L. yucatanensis* n. sp. Furthermore, the penis enters the accessory piece proximally in *L. huitrempe* vs. distally in *L.*

yucatanensis n. sp. The anchors differ in shape and size, and the ventral bar shows a V-shaped knot in *L. huitrempre*, whereas the knot is absent and *L. yucatanensis* n. sp.

Finally, *L. yucatanensis* n. sp. is also quite similar to *Ligophorus triangularis* Sarabeev V., Rubtsova N., Yang T., Balbuena J.A., 2013, which occurs on *Liza haematocheila* Temminck and Schlegel, 1845 in the Sea of Japan. *L. yucatanensis* n. sp. resembles *L. triangularis* in the C-shaped, elongated, thin copulatory organ and the claw-shaped pincerlike accessory piece. However, they differ in the morphology of the main lobe of the accessory piece, which is cylindrical bowed, elongated with a subtriangular heavy-sclerotized expansion at its distal part in *L. triangularis*, whereas it is cylindrical, tunnelled expanded distally, slightly bowed with a characteristic membranous opening at the level of the medial bifurcation of the accessory piece, reaching to a thick-walled bulb-shaped expansion and terminating in a round "labium" in *L. yucatanensis* n. sp. In addition, both species also differ in the distal end of vagina, which is funnel-shaped in *L. triangularis* and scyphoid and narrow in *L. yucatanensis* n. sp.

The morphometric comparison with species of *Ligophorus* from the Gulf of Mexico and Caribbean Sea (Table 5.1) indicates that the body length of *L. yucatanensis* n. sp. is smaller and the ventral anchors are shorter (VAA, VAB and VAG) than those of the other species in the area.

5.3. Discussion

Ligophorus yucatanensis n. sp. is added to the 60 species currently recognized in *Ligophorus* in the world. No species of *Ligophorus* have been reported from the Yucatan Peninsula and the present work represents the first record of the genus in Mexico and provides the morphological description of a new species on *M. cephalus* sensu lato.

The species of *Ligophorus* previously recorded in the Americas in different hosts are *L. mugilinus* and *L. huitrempe* Fernández-Bargiela 1987 from *M. cephalus*; *L. tainhae*, *L. brasiliensis*, *L. guanduensis* and *L.*

lizae Abdallah, Azevedo and Luque 2009 on *M. liza* Valenciennes 1936; and *L. saladensis* Marcotegui and Martorelli 2009 and *L. uruguayense* Failla and Ostrowski de Núñez 2009 on *M. platanus* Günther 1880. Note that records of *L. mugilinus* on *M. curema* need confirmation (Sarabeev et al. 2005). The results presented herein show that the new species resembles more closely species from Mediterranean Sea and off the coast of the northwestern Pacific than the species recorded in South and North America as it reported for the species in Brazil (Abdallah et al. 2009).

The prevalence of *L. yucatanensis* n. sp. observed herein (77%) is in the range of that reported for *Ligophorus* spp. from the Caribbean (48–100%) on white mullets *M. curema* (Sarabeev et al. 2005). Interestingly previous studies on *Ligophorus* have shown that the species display a strict host specificity in that each host species is infected by a combination of *Ligophorus* spp. that are not found on other mullets (Marcotegui and Martorelli, 2009).

In addition, some authors have suggested that, due to the coastal preference of mugilids, the open ocean has acted as a geographical barrier favouring the speciation of monogeneans within grey mullet populations (Sarabeev et al. 2005; Marcotegui and Martorelli, 2009). Therefore, the analysis of records of *Ligophorus* spp. on *M. cephalus* may help as indicators to determine the assemblage of species that constitute the *M. cephalus* complex (Marianello et al. 2004; El Hafidi et al. 2013a).

El Hafidi et al. (2013a) reviewed the zoographic records of *Ligophorus* spp. from the *M. cephalus* species complex. The authors recognized 14 geographical host entities, and the new species described herein is added to entity 4: Northwest Atlantic Ocean (Figure 5.4), where only *L. mugilinus* had been so far recorded. Although generally correct, their zoogeographic account needs to be updated in order to include data not available at the time of their publication (Figure 5.4).

The recently described *L. abditus* is added to host entity 13 (Sea of Japan). *L. chongmingensis* Hu and Li 1992 and *L. leporinus* Zhang and Ji 1981, both assigned to entity 14, represent species inquirendae, whose adscription to *Ligophorus* is questionable (Sarabeev et al. 2013).

Table 5.1 Morphometric measurements (Mean ± standard deviation and range in parenthesis) of the haptoral and copulatory hard-parts of *Ligophorus yucatanensis* n. sp. and other *Ligophorus* spp. recorded in the Gulf of Mexico and the Caribbean Sea.

Characters[a]	Present study *Ligophorus yucatanensis* n. sp. ex *Mugil cephalus* From Celestun Lagoon Yucatan, Mexico		Sarabeev *et al.* (2013) *Ligophorus mugilinus* ex *Mugil cephalus* From Northwest Atlantic, Charleston coastal waters, USA		Sarabeev *et al.* (2005)[b] *Ligophorus mugilinus* ex *Mugil cephalus* From Gulf of Mexico, Alligator Harbor		Sarabeev *et al.* (2005)[c] *Ligophorus* sp. ex *Mugil curema* Caribbean Sea, Punta Santiago Puerto Rico		Sarabeev *et al.* (2005)[d] *Ligophorus* sp. ex *Mugil curema* Caribbean Sea, Margarita Island, Venezuela
	Mean ± SD (Min-Max)	N	Mean ± SD (Min-Max)	N	Mean ± SD (Min-Max)	N	Mean ± SD (Min-Max)	N	Mean N=1
Body length	514 ± 47.7 (467-577)	3	702 ± 93.1 (616-806)	5	600 ± 27 (569-616)	3	861 ± 212 (711-1011)	2	687
Body width	130 ± 38.5 (97-185)	3	115 ± 17.4 (100-142)	5	107 ± 6 (100-11)	3	122 ± 28 (103-142)	2	142
VAA	31 ± 1.2 (29-33)	10	37 ± 1.3 (36-39)	5	35 ± 1 (33-35)	3	40 ± 4 (37-43)	2	25
VAB	19 ± 1.3 (17-21)	10	25 ± 1.0 (24-27)	5	24 ± 2 (22-25)	3	33 ± 4 (30-37)	2	14
VAC	8 ± 1.4 (5-10)	10	9 ± 1.0 (8-10)	5	8 ± 0.4 (8-9)	3	10 ± 1 (9-1)	2	8
VAD	19 ± 1.0 (17-20)	10	18 ± 0.9 (17-19)	5	17 ± 2 (16-19)	3	18 ± 5 (15-22)	2	16
VAE	8 ±0.8 (7-10)	10	9 ± 0.6 (8-10)	5	9 ± (9)	3	12 ± 0.2 (12)	2	14
VAF	13 ± 0.9 (12-14)	10	17 ± 2.3 (14-18)	3	-	-	-	-	-
BL	26 ± 1.1 (25-28)	10	-	-	-	-	-	-	-
VAG	27 ± 1.0 (25-28)	10	34 ± 1.5 (32-36)	5	-	-		-	-
DAA	38 ±1.0 (37-40)	10	41 ± 1.3 (39-42)	5	38 ± 2 (35-40)	3	39 ± 1 (39-40)	2	36
DAB	26 ± 2.4 (20-28)	10	28 ± 0.9 (27-29)	5	25 ± 1 (24-27)	3	27 ± 3 (25-30)	2	27
DAC	9 ± 2.3 (5-12)	10	9 ± 0.5 (9-10)	5	8 ± 1 (9)	3	11 ± 1 (11-12)	2	13
DAD	18 ± 1.2 (16-20)	10	18 ± 0.7 (17-19)	5	17 ± 1 (16-18)	3	19 ± 4 (16-21)	2	16
DAE	8 ± 0.3 (8-9)	10	10 ±0.3 (9-10)	5	9 ± 0 (9-10)	3	10 ± 0.4 (10-11)	2	8
DAF	17 ± 1.3 (15-20)	10	20 ± 2.5 (17-22)	3	-	-	-	-	-
DAG	36 ± 1.3 (33-38)	10	37 ± 1.1 (35-38)	5	-	-	-	-	-
HTL	11 ± 1.3 (9-13)	10	12 ± 0.7 (11-13)	5	-	-	-	-	-
VBL	42 ± 2.5 (39-47)	10	39 ± 2.8 (37-43)	5	41 ± 2 (39-43)	3	44 ± 2 (42-45)	2	42
VBDP	7 ± 0.8 (5-8)	10	8 ± 2.4 (5-11)	5	-	-		-	-
DBL	40 ± 3.3 (36-47)	10	35 ± 2.9 (32-39)	5	-	-	51 ± 4 (47-54)	2	38
APTL	26.4 ± 2.7 (24.5-29.2)	6	30 ± 2.6 (27-33)	5	-	-	-	-	-
APML	12.8± 2.1 (10-14.6)	6	23 ± 2.6 (20-25)	3	-	-	-	-	-
APMW	5 ± 1.2 (3-7)	10	3 ± 0.6 (3-4)	3	-	-	-	-	-

APSL	6 ± 1.8 (3-9)	10	22 ± 0.6 (22-23)	3	-	-	-	-	-
COL	93 ± 9.9 (75-103)	10	79 ± 4.7 (73-85)	5	-	-	-	-	-
COW	1 ± 0.3 (0-1)	10	0.9 ± 0.2 (0.7-1)	3	-	-	-	-	-
VL	31 ± 7.7 (23-41)	4	44 ± 5.9 (35-51)	5	-	-	-	-	-

[a] See Material and methods section for abbreviations of metric variables of sclerotized characters.
[b] Measurements based on new specimens and holotype and paratypes of Hargis's (1955).
[c, d] Reported as *L. mugilinus*. Doubtful species (require additional information).

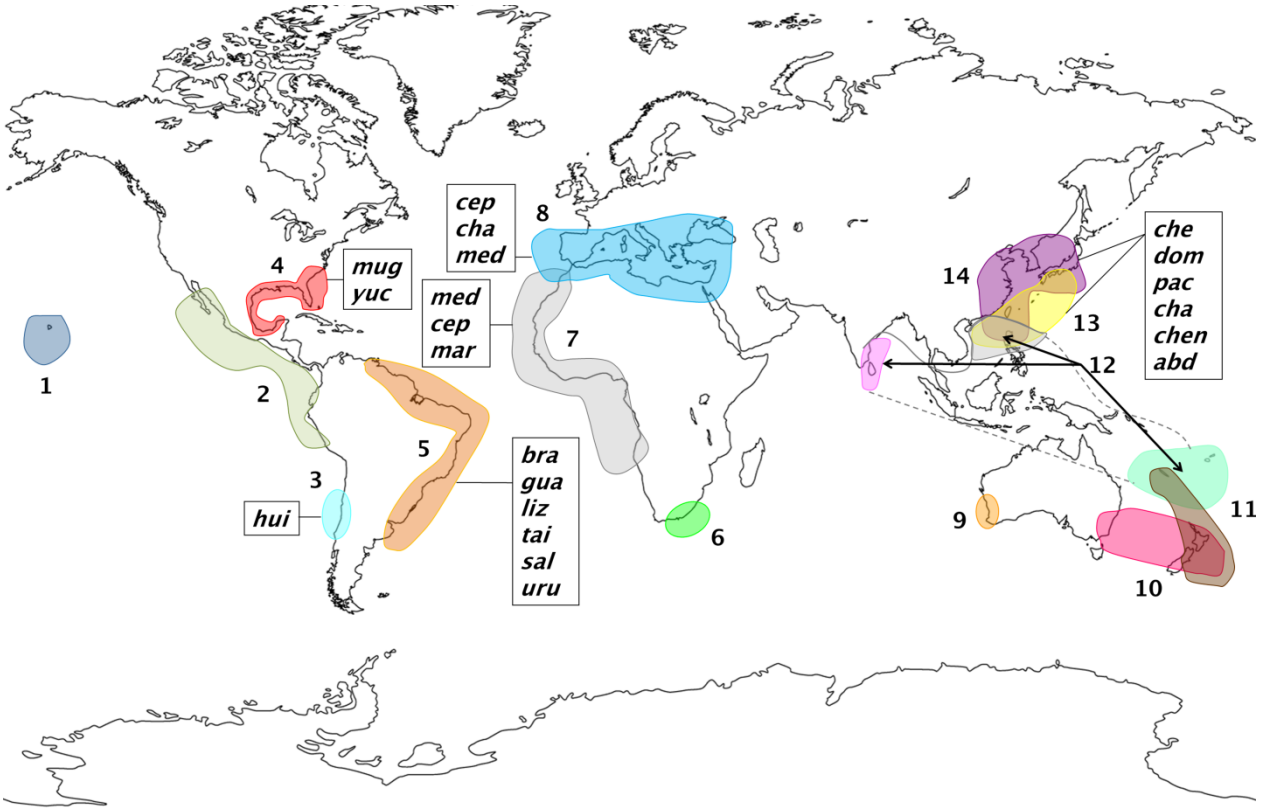

Figure 5.4. Records of *Ligophorus* spp. on 14 genetically distinct entities of *Mugil cephalus* (Modified from Whitefield et al. 2012 and El Hafidi et al. 2013a): (1) Hawaii; (2) Central East Pacific; (3) Southern East Pacific; (4) Western North Atlantic; (5) Western Central Atlantic; (6) Southern Africa; (7) Western Africa; (8) Mediterranean Sea; (9) Western Australia; (10) South Eastern Australia; (11) Western Pacific; (12) Central Western Pacific and North West Indian Ocean; (13,14) Sea of Japan, Yellow Sea and East China Sea. Abbreviations: *hui*: *L. huitrempe*, *mug*: *L. mugilinus*, *yuc*: *L. yucatanensis n. sp.*, *cep*: *L. cephali*, *med*: *L. mediterraneus*, *cha*: *L. chabaudi*, *mar*: *L. maroccanus*, *che*: *L. cheleus*, *dom*: *L. domnichi*, *abd*: *L. abditus*, *pac*: *L. pacificus*, *che*: *L. chenzhenensis*, *bra*: *L. brasiliensis*, *gua*: *L. guanduensis*, *liz*: *L. lizae*, *tai*: *L. tainhae*, *sal: L. saladensis*, *uru*: *L. uruguayense*. Only species that occur typically on *M. cephalus* sensu lato have been considered.

In addition, the record of *L. mugilinus* in the East China Sea (entity 14) is most probably incorrect, given the substantial morphological differences between this form and specimens of *L. mugilinus* from its area of origin (Sarabeev et al. 2013). Therefore, only one species of *Ligophorus*, *L. chabaudi*, has been reported from two geographically distant areas (entities 8 and 14, Figure 5.4). Altogether, this evidence supports El Hafidi et al. (2013a) hypothesis that different species of *Ligophorus* infect the different entities composing the *M. cephalus* species complex. The implicit assumption is that *Ligophorus* spp. have a limited dispersal ability and only hosts from overlapping (e.g. entities 13 and

14) or contiguous (e.g. entities 7 and 8) populations can share species. Therefore, the presence of *L. chabaudi* in two distant host entities suggests that there might be two geographically- segregated cryptic species (El Hafidi et al. 2013a) and clearly calls for a re-evaluation of the Pacific forms of *L. chabaudi*.

Figure 5.4 suggests that most *Ligophorus* species on the *M. cephalus* complex are found in the Northern Hemisphere, this might merely reflect differences in sampling effort, since several southern host entities (e.g. 6, 9–11) have been hardly surveyed for parasites.

In addition, more local surveys are also required. In Mexico, for instance, grey mullets are an important gill net fishery resource along both the Pacific and Gulf of Mexico coasts. The fisheries of *M. cephalus* and *M. curema* in the Gulf of Mexico are concentrated on the north and central-western side, especially in river-estuarine systems, coastal lagoons and offshore spawning areas. In the southern coast the fisheries are less important (Meléndez-Galicia and Romero-Acosta, 2010). The only species of *Ligophorus* reported so far is *L. yucatanensis* n. sp. in the southern coastal Celestun Lagoon. Although we tried to support the present morphological evidence with molecular analyses, DNA could not be amplified due to the condition and low number of the specimens available.
Thus further parasitological surveys in the Gulf of Mexico are needed to obtain additional morphological and molecular evidence for this and possibly other undiscovered species of the genus.

It is clear that further studies on *M. cephalus* sensu lato in relation with environmental factors are needed in order to better understand the speciation patterns of *Ligophorus* and processes connected with the evolution of host specificity in congeneric monogeneans parasitizing grey mullets. For instance, Marchiori et al. (2015) suggested that *L. saladensis* and *L. uruguayense* from the closely related to *M. cephalus* host *M. liza*, represent a complex of closely related species with *L. mediterraneus*. The presence of separate but closely related parasites species on these closely related hosts lends

credit to the suggestion that species of *Ligophorus* can be used as a marker for taxonomy and evolution of mullet species (El Hafidi et al. 2013a). Finally, we conclude that *L. yucatanensis* n. sp. should be erected as a new species based on its morphological differences, distinct from others in the genus and the dissimilar geographical distribution of the genetically different entities of *M. cephalus*.

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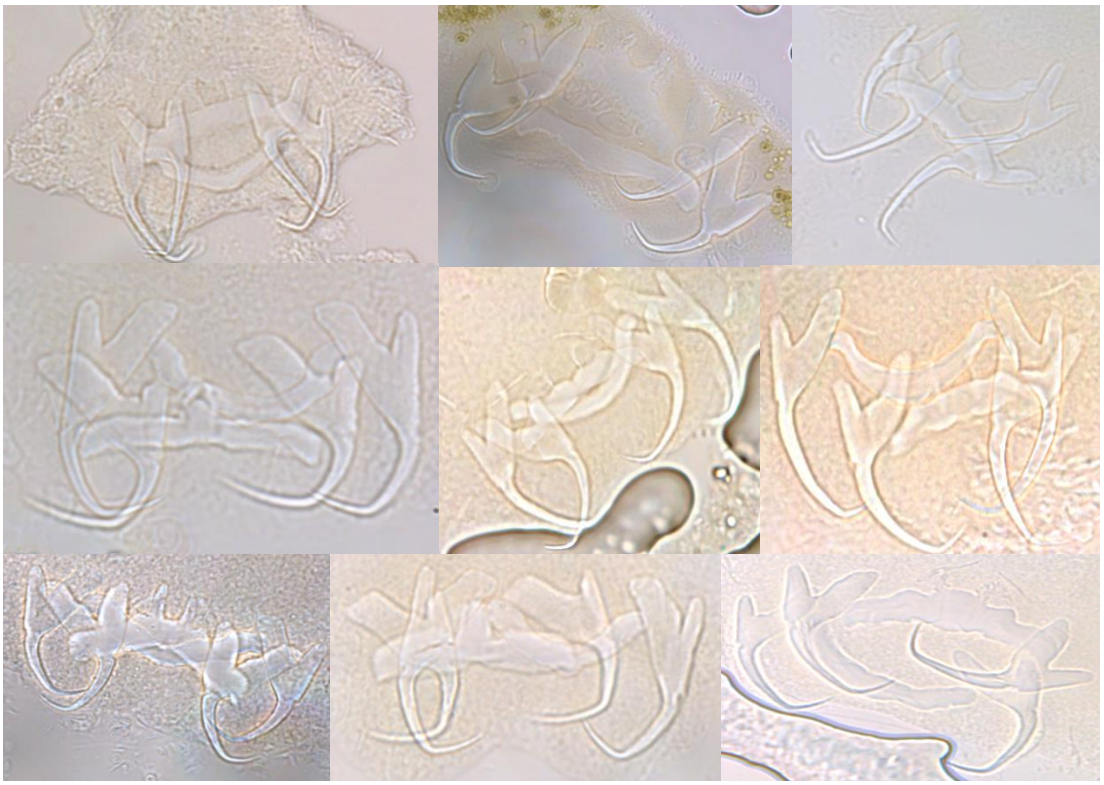
CHAPTER 6

Evolutionary modularity and morphological integration in the haptoral anchor structures of *Ligophorus* spp. (Monogenea: Dactylogyridae)



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Abstract

An important question in the study of phenotypic evolution is whether characters are independent of each other or behave and evolve as integrated modules. Morphological integration and modularity provide a powerful framework for the analysis of the evolution of morphological traits. We used geometric morphometrics and phylogenetically independent contrasts (PIC) to test four different modularity hypotheses in the haptoral anchors of 14 monogenean species of *Ligophorus*. Integration between the modular units identified was further evaluated with two-block partial least squares analysis. Roots and points represented two modules in the dorsal and ventral anchors, but modularity was not statistically supported when parasite phylogeny was accounted for, which may indicate convergent evolution related to host characteristics and gill morphology. In contrast, PIC revealed medial and lateral modules in ventral anchors only. Moreover, we found evidence for ventral and dorsal anchor pairs forming two modules, supporting the notion that they play different functional roles. Integration between all identified modules was strong. We conclude that there is modular structure in the anchors of *Ligophorus* spp., accounted by adaptive and phylogenetic factors acting at different levels, and ventral and dorsal anchors evolve as integrated modules with specific roles in attachment.

Keywords *Ligophorus* spp., Haptor, Morphological integration, Modularity, Morphological evolution.

6.1. Introduction

Most organisms and structures can be split up into recognizable, relatively independent parts that are coherent according to their developmental origin, structure, and/or function. Nevertheless, it is also clear that this independence is far from complete because the parts of organisms are coordinated among one another and integrated throughout the whole organism. These ideas about coordination and independence of organismal parts are encapsulated in the concepts of integration and modularity, respectively (Klingenberg, 2008).

Morphological integration focuses on the connection between or among an organism's morphological traits, related functionally, developmentally, genetically, and/or evolutionarily (Klingenberg, 2008). It has been quantified applying several methods, which enable the estimation of the covariation among the sets of traits (or, alternatively, of an entire morphological structure; Sanger et al. 2011). Morphological integration is manifested at the macroevolutionary level, where it reflects the way in which evolutionary changes in different parts of organisms are associated (Klingenberg and Marugán-Lobón, 2013) and may result from coordinated selection or from drift of genetically correlated traits (Klingenberg, 2010) and could constrain the variability in individual traits and facilitate modifications of these traits.

Morphological integration is not uniform throughout entire organisms, but tends to be concentrated in certain complexes of parts that are tightly integrated internally, but are relatively independent of other such complexes. Such complexes are called "modules" (Klingenberg, 2008). These modules are defined with the general aim of understanding how structures are morphologically organized, how they vary, and, ultimately, how they evolve (Klingenberg, 2010; Arias-Martorell et al. 2014) and can be assessed by analyzing the strength of association between subsets of landmarks in a configuration (Klingenberg, 2009). At the macroevolutionary level, modularity refers to complexes of traits that evolve in relative independence of each other (Klingenberg and Marugán-Lobón, 2013), is the result of correlated evolution in distinct sets of

traits (Klingenberg, 2008; Jojić et al. 2012), and is expected to play a significant role in the evolution of complex morphologies (Sanger et al. 2011). The potential of this approach to make functional inferences of organismal forms has been shown in Drake and Klingenberg (2010), Klingenberg and Marugán-Lobón (2013), and Benítez et al. (2014), among others.

Because phylogenetically related species tend to resemble each other in many aspects of their phenotype, as well as in ecological characteristics, more than expected by pure chance, they cannot be considered to represent independent observations (Hernández et al. 2013). Consequently, the study of integration and modularity at the evolutionary level requires the adoption of comparative approaches (Harvey and Pagel, 1991) that take into account the phylogenetic relations in the structure of the data. This solves the statistical problem of non-independence of the observations and removes the effect of shared ancestry on the variability in characters such that taxa are statistically independent (Harvey and Pagel, 1991).

Parasites seem to represent ideal targets for comparative studies because of their evident putative adaptive features and their intricate relationships with their host, which themselves represent a well-defined resource (e.g., environment) tractable through evolutionary time via phylogenetic tree (Morand et al. 2015). Comparative methods have been applied to the study of evolution of host specificity in *Lamellodiscus* spp., parasite evolutionary ecology, diversification, diversity, and community ecology, as well as to establishing the link between host specificity and species richness within a monogenean (Morand and Poulin, 1998, 2003; Desdevises et al. 2001, 2002; Webb et al. 2002). However, their application in a geometric morphometric context has remained largely unexplored. More specifically, only a few studies have focused on modularity and integration in the attachment of monogeneans (Vignon et al. 2011; Rodríguez-González et al. 2015; Khang et al. 2016).

In the present study, we use the haptoral anchors of monogenean species of *Ligophorus* as a model system. This model is interesting because *Ligophorus* is a speciose taxon, whose species are

oioxenous and restricted to grey mullets (Mugilidae), and several congeneric species can coexist on the same host (Blasco-Costa et al. 2012; Sarabeev et al. 2013; Sarabeev and Desdevises, 2014). In addition, it has been shown that the morphological variability of hard structures in this genus supports the validity of morphometric characters (Blasco-Costa et al. 2012). In *Ligophorus*, the anchors show morphological plasticity probably as a result of host-induced plastic responses (Rodríguez-González et al. 2015; Llopis-Belenguer et al. 2015), and their morphology plays a major role in taxonomy (Sarabeev et al. 2013). In fact, the haptoral sclerotized structures are extremely diverse and are used for a secure and permanent attachment on gills and considered as the "hallmark" for monogeneans (Wong and Gorb, 2013). In monogeneans, this diversity includes different fixation structures, such as marginal hooks, suckers, clamps, squamoid discs, adhesive secretions, and anchors (Wong and Gorb, 2013). The present study focuses on the morphology of anchors.

Given that attachment is crucial for survival in monogeneans, the morphological study of the attachment organs plays an important role in specialization and adaptation to host species (Šimková et al. 2001, 2002; Vignon et al. 2011), providing valuable cues to understand these processes; therefore, the shape of the haptor should represent an important feature connected with host specificity. This host specificity may be determined by host predictability (hypothesis of specialization on predictable resources) and can be linked to parasite distribution (hypothesis of ecological specialization; Mendlová and Šimková, 2014). Examples of this specialization have been observed in the anchors of *Dactylogyrus*, where the total length and the length of base of anchor correlate with the host body size, suggesting that specialization is related to the adaptation of the haptor to the host (Šimková et al. 2001). Likewise, in Lamellodiscus, the relationship between host size and parasite body size has been interpreted as evidence of an underlying mechanism optimizing the morphological adaptation to host species (Desdevises et al. 2002).

Although the morphology of the haptor and the similarity of the anchors among species have been interpreted as an outcome of adaptive processes related to the ecology or the morphology of the host, other authors maintain that haptoral morphology is a reflection of phylogenetic constraints (Khang et al. 2016). The debate is far from being settled. Whereas several studies suggest that haptor morphology correlates with parasite phylogeny (Desdevises et al. 2002; Vignon et al. 2011; Mendlová and Šimková, 2014), others suggest that the morphology of the haptor represents an important adaptation of parasites to their hosts (host specificity) and to specific sites within their hosts (niche preference; Šimková et al. 2002, 2006; Messu Mandeng et al. 2015). The modular organization in the haptor structures and the degree of covariation between parts of a structure (e.g., roots and point of anchors) can help understand how phylogenetic and adaptive processes interact (Vignon et al. 2011; Khang et al. 2016). However, the relations of modularity between haptoral parts are still not well understood (Vignon et al. 2011).

Recent studies have shown functional differences and high level of morphological integration between roots and points in both anchors, indicating a concerted action between them in species of *Ligophorus*, supporting the notion of a tight integration between the root and the point compartments as a single and fully integrated module (Rodríguez-González et al. 2015; Khang et al. 2016). However, these studies did not evaluate the modularity and neither relationship with effect of phylogeny. In this paper, we studied the ventral and dorsal anchors of 14 species of *Ligophorus* to address the question whether morphological variation of the anchors is integrated or whether anchor regions evolve as distinct modules. If modules were identified, strong integration among parts may influence patterns of variability of anchor shapes among congeneric species as well as the direction of evolution under selection (Khang et al. 2016).

Therefore, the aim of this study was to determine whether variation in the anchor shapes in *Ligophorus* spp. is modular and integrated. If this were the case, anchor shape could be constrained by either phylogeny or convergent evolution. To examine this issue, we used geometric

morphometric methods to quantify the modular variation of anchor shape in an explicit phylogenetic framework by means of phylogenetically independent contrasts (PIC) at the macroevolutionary level (Klingenberg and Marugán-Lobón, 2013). Using a multivariate correlation coefficient adapted for modularity assessment called the *RV* coefficient (Klingenberg, 2009) and exploring the morphological integration using a two-block partial least squares analysis, we identify the partitions of the anchors in four integrated compartments that agree well with information on musculature arrangements (Petrov et al. 2015).

6.2. Material and methods

6.2.1. Ethical statement

The fishes needed for the study were obtained within day-today fishery operations and purchased dead from licensed commercial fishermen. The number of specimens used (77) was kept to a reasonable minimum to guarantee the success of the research (see Table 6.1). Grey mullets are locally and globally abundant and are not subjected to special conservation regulations in Spain, and the species involved—*Mugil cephalus* L. (flathead mullet), *Liza saliens* (Risso; leaping mullet), *Liza ramada* (Risso; thin-lipped grey mullet), *Liza aurata* (Risso; golden grey mullet), *Chelon labrosus* (Risso; thicklip grey mullet), and *Liza haematocheila* (Temminck et Schlegel; soiuy mullet)—are listed by the IUCN as "Least Concern".

6.2.2. Data acquisition

This study covers all grey mullet species reported as host of *Ligophorus* spp. in the Mediterranean, Black Sea, and Sea of Azov, including *Lz. haematocheila*, which was introduced in the Black Sea and Sea of Azov from the Pacific in the early 1980s (Sarabeev, 2015), and all 14 species of *Ligophorus* (about 23 % of all known species of the genus) recorded in the study area. This includes *Ligophorus*

llewellyni Dmitrieva et al. 2007 and *Ligophorus pilengas* Sarabeev & Balbuena, 2004, both occurring on the introduced so-iuy mullet, although part of the specimens studied herein came from the native Pacific waters (Table 6.1).

The species of *Ligophorus* are specific gill parasites of grey mullets (Sarabeev et al. 2013). The haptor in *Ligophorus* spp. consists of seven pairs of marginal hooks and two pairs of anchors (dorsal and ventral), which are connected by respective transversal dorsal and ventral bars (Euzet and Suriano, 1977), and the anchors and bars are primarily involved in attachment to the gill, while the marginal hooks are minute and do not play a significant role in the attachment of adult specimens (Petrov et al. 2015).

We based our morphological analysis on 286 individuals belonging to 14 of 16 valid species of *Ligophorus* for which original drawings of anchors were available: *Ligophorus acuminatus* Euzet & Suriano, 1977; *Ligophorus cephali* Rubtsova et al. 2006; *Ligophorus chabaudi* Euzet & Suriano, 1977; *Ligophorus confusus* Euzet & Suriano, 1977; *Ligophorus heteronchus* Euzet & Suriano, 1977; *Ligophorus imitans* Euzet & Suriano, 1977; *Ligophorus macrocolpos* Euzet & Suriano 1977; *Ligophorus mediterraneus* Sarabeev et al. 2005; *Ligophorus minimus* Euzet & Suriano, 1977; *Ligophorus szidati* Euzet & Suriano, 1977; *Ligophorus vanbenedenii* Euzet & Suriano, 1977; *L. llewellyni*; *L. pilengas*, and *Ligophorus angustus* Euzet & Suriano, 1977. The sample size for each species was 20 individuals for ventral and 20 individuals for dorsal anchors (not always matching specimens of the previous group), except in *L. angustus* (four ventral and two dorsal anchors). In all, 526 anchors were studied of which, in 238 instances, represented ventral and dorsal anchors of the same worm individual. The specimens were collected in two marine areas of the Spanish Mediterranean Coast (the Ebro Delta and Santa Pola Bay), a coastal Mediterranean lagoon (L'Albufera), and the Sea of Azov (Kerch Strait) and Sea of Japan (Artemovka Delta; Table 6.1). Details of how gills were examined for parasites are given in Míguez-Lozano et al. (2012) and Rodríguez- González et al. (2015).

We used photographs and drawings only for ventral and dorsal anchors of partly digested individuals, following Rodríguez-González et al. (2015). Specifically, one anchor from each pair (left or right) from each different specimen was chosen for analysis, as well as one pair of anchors (ventral–dorsal) for species. Only the anchors (i.e., ventral and dorsal, from each specimen) on both sides were considered for geometric morphometric techniques because they are not subject to large variation due to contraction or flattening on fixation (Lim and Gibson, 2009). The bars were not studied because they are more difficult to observe flat and more prone to distortion during fixation and mounting. Specifically, one anchor from each pair (left or right) from each different specimen was chosen for analysis (Rodríguez-González et al. 2015). The anchors were drawn using a drawing tube at x 100 (under immersion oil) under a Nikon Optiphot-2 microscope equipped with interference contrast.

6.2.3. Morphometric analysis

The analyses were based on a two-dimensional geometric morphometric approach (Zelditch et al. 2012). This technique allows analyzing separately the two components of variation of forms, i.e., size and shape, and visualizing the results as shape changes of specific regions of the biological structures under examination. Raw images of the anchors of all specimens of *Ligophorus* were compiled and scaled with tpsUtil version 1.52 (Rohlf, 2012). Eight landmarks were digitized using tpsDig2 version 2.17 (Rohlf 2013, available at http://life.bio.sunysb.edu/morph/). The criteria and description used for the landmark assignation in the ventral and dorsal anchors in all the species were according to Rodríguez-González et al. (2015) (see Figure 4.1).

For each species, landmark configurations for both ventral and dorsal anchors were superimposed using a generalized Procrustes analysis (GPA), which removes differences in scaling, rotation, and translation (Rohlf and Slice, 1990; Bookstein, 1996) and superimposes the corresponding landmarks. After superimposition, GPA provides two new sets of variables of form:

the pure shape of anchors, which is represented by coordinates of the aligned landmarks, and size (centroid size), computed as the square root of the summed distances between each landmark coordinate and the centroid configuration (Zelditch et al. 2012). In this study, to control the effect of size on shape (allometry), we used lnCS (log-transformed centroid size) as a measure of anchor size (Klingenberg et al. 2012) because it produces a better fit of linear relationship, which is estimated by the percentage of shape variance explained by size (Drake and Klingenberg, 2008).

Table 6.1 Species of *Ligophorus* used in this study collected from five localities: Ebro Delta (40°30′–40°50′N, 0°30′–1°10′E); Santa Pola Bay (38°00′–38°20′N, 0°10′–0°40′W); L'Albufera (39°20′0″N–0°21′0″W); Kerch Strait, Sea of Azov (45°16′20.8″N–36°31′40.6″E); and Artemovka Delta, Sea of Japan (43°18′30.3″N–132°17′4.8″E).

Species of *Ligophorus*	N	Host species	Host size, *N* (Mean ± SD)	Date	Ebro Delta	Santa Pola	L'Albufera	Kerch Strait	Artemovka Delta
Ligophorus acuminatus Euzet and Suriano, 1977	20	*Liza saliens*	7 (492.05 ± 283.16)	Spring 2004 Autumn 2005	X				
Ligophorus cephali Rubtsova, Balbuena, Sarabeev, Blasco-Costa and Euzet, 2006	20	*Mugil cephalus*	20 (409.10 ± 147.88)	Autumn 2011 Spring 2014			X		
Ligophorus chabaudi Euzet and Suriano, 1977	20	*Mugil cephalus*	4 (622.47 ± 109.66)	Spring 2005 Spring 2014	X	X			
Ligophorus confusus Euzet and Suriano, 1977	20	*Liza ramada*	5 (1358.91 ± 568.06)	Spring 2014		X			
Ligophorus heteronchus Euzet and Suriano, 1977	20	*Liza saliens*	3 (509.23 ± 173.86)	Spring 2004 Autumn 2005	X				
Ligophorus imitans Euzet and Suriano, 1977	20	*Liza ramada*	3 (607.31 ± 78.72)	Autumn 2005	X	X			
Ligophorus macrocolpos Euzet and Suriano, 1977	20	*Liza saliens*	4 (192.01 ± 90.85)	Spring 2004 Autumn 2005	X	X			
Ligophorus mediterraneus Sarabeev, Balbuena and Euzet, 2005	20	*Mugil cephalus*	3 (541.21 ± 89.18)	Spring 2005 Spring 2014	X	X			
Ligophorus minimus Euzet and Suriano, 1977	20	*Liza saliens*	7 (443.69 ± 372.71)	Spring 2004 Autumn 2005 Spring 2014	X		X		
Ligophorus szidati, Euzet and Suriano, 1977	20	*Liza aurata*	4 (584.35 ± 201.08)	Autumn 2005 Spring 2014		X			
Ligophorus vanbenedenii Euzet and Suriano, 1977	20	*Liza aurata*	5 (608.45 ± 316.13)	Spring 2004 Autumn 2005 Spring 2014		X			
Ligophorus llewellyni Dmitrieva, Gerasev and Pron'kina, 2007	20	*Liza haematocheila*	4 (31.67 ± 3.80)	Spring 2005 Summer 2005				X	X
Ligophorus pilengas Sarabeev and Balbuena, 2004	20	*Liza haematocheila*	5 (35.34 ± 8.83)	Spring 2004 Spring 2005 Summer 2005 Autumn 2005				X	X
Ligophorus angustus Euzet and Suriano, 1977	6	*Chelon labrosus*	3 (710.33 ± 198.58)	Spring 2004 Autumn 2005	X				

N sample size (for ventral and dorsal anchors), SD standard deviation

All geometric morphometric analyses were performed with MorphoJ version 1.06d (Klingenberg, 2011, available at http://www.flywings.org.uk/MorphoJ_page.htm).

6.2.4. Phylogeny and comparative approach

To obtain a phylogeny of the species of *Ligophorus*, the 28S rDNA and ITS1 sequences of the Mediterranean species of Blasco-Costa et al. (2012) available in GenBank (Table 6.2) were used. *Ergenstrema mugilis* Paperna 1964 was used as the outgroup. The sequences of each gene were aligned using MUSCLE (Edgar 2004) in MEGA v.6.0 (Tamura et al. 2013) and corrected by eye. The alignments of 28S and ITS1 sequences comprised 866 and 779 positions, respectively. The nucleotide substitution model for phylogenetic reconstruction was estimated independently for each dataset using jModelTest v. 2.1.6 (Darriba et al. 2012). The model GTR+I+ Γ was found to fit the two datasets best on the basis of its Akaike's information criterion score, with a gamma shape parameter (alpha) of 0.63 for the 28S gene and 0.62 for ITS1 and values for the proportion of invariable sites of 0.53 and 0.17, respectively. The aligned sequences from the two genes were concatenated using Sequence Matrix v.1.8 (Vaidya and Meier, 2011). Bayesian inference (BI) using MrBayes v. 3.2.2 (Ronquist et al. 2012) and maximum likelihood (ML) using PhyML ver. 3.0 (Guindon et al. 2010) were used for phylogenetic reconstruction, dealing with both genes as independent partitions estimating all the parameters independently. For the ML analysis, a starting tree was built based on neighbor joining, followed by two methods of tree searching-nearest neighbor interchange and subtree pruning and regrafting-returning the best solution among them. Branch support was estimated by bootstrap analysis with 1000 replicates. BI analysis was performed with four Markov chain Monte Carlo ran for 10^6 generations with a sampling frequency of 1000 and a "burn-in" set value of 25 % of the stored trees. Stationarity of the Markov chain was reached at 10^6 generations, evidenced by a standard deviation of split frequencies <0.01 and by a potential scale reduction factor converging to 1. A majority rule consensus tree was built after

discarding the first 25 % of the trees ("burn-in" set). The nodal support was estimated as posterior probabilities (Huelsenbeck et al. 2001). The topology obtained is shown in the supplementary material (Figure 6S1).

Table 6.2 Modified table of Blasco-Costa et al. (2012) with additional sampling locations of *Ligophorus* spp. and *E. mugilis* (outgroup) sequenced, their hosts, locality, and GenBank accession no. for sequences.

Species of *Ligophorus* sequenced	Hosts	Locality	GenBank accession numbers	
			28S rDNA	ITS-1
Ligophorus acuminatus Euzet et Suriano, 1977	*Liza saliens*	Ebro Delta[a]	JN996816	JN996852
Ligophorus angustus Euzet et Suriano, 1977	*Chelon labrosus*	Off Cullera[a]	JN996804	JN996839
Ligophorus cephali Rubtsova, Balbuena, Sarabeev, Blasco-Costa et Euzet 2006	*Mugil cephalus*	L'Albufera Lagoon, Off Cullera[a]	JN996830	JN996865
Ligophorus chabaudi Euzet et Suriano, 1977	*Mugil cephalus*	Santa Pola, Ebro Delta[a]	JN996831	JN996866
Ligophorus confusus Euzet et Suriano, 1977	*Liza ramada*	Santa Pola, Off Cullera[a], Ebro Delta[a]	JN996808	JN996843
Ligophorus heteronchus Euzet et Suriano, 1977	*Liza saliens*	Ebro Delta[a]	JN996812	JN996848
Ligophorus imitans Euzet et Suriano, 1977	*Liza ramada*	Santa Pola, Ebro Delta[a]	JN996813	JN996849
Ligophorus llewellyni Dmitrieva, Gerasev et Pron'kina, 2007	*Liza haematocheila*	Utlyuksky Estuary[a]	JN996822	JN996858
Ligophorus macrocolpos Euzet et Suriano, 1977	*Liza saliens*	Ebro Delta[a]	JN996819	JN996855
Ligophorus mediterraneus Saraveeb, Balbuena et Euzet, 2005	*Mugil cephalus*	Santa Pola, Ebro Delta, Off Cullera[a]	JN996828	JN996863
Ligophorus minimus Euzet et Suriano, 1977	*Liza saliens*	L' Albufera, Ebro Delta[a]	JN996817	JN996853
Ligophorus pilengas Sarabeev et Balbuena 2004	*Liza haematocheila*	Utlyuksky Estuary[a]	JN996824	JN996859
Ligophorus szidati Euzet et Suriano, 1977	*Liza aurata*	Santa Pola, Ebro Delta[a]	JN996806	JN996841
Ligophorus vanbenedenii Euzet et Suriano, 1977	*Liza aurata*	Santa Pola, Ebro Delta[a]	JN996802	JN996837
Ergenstrema mugilis (outgroup) Paperna, 1964	*Liza ramada*	Ebro Delta[a]	JN996800	JN996835

[a] Localities for the sequenced *Ligophorus*

The comparative analyses in this study are based on the phylogenetic tree of *Ligophorus* spp. We used independent contrasts as the method for taking into account the phylogenetic nature of the comparative data (Felsenstein, 1985). Unlike other methods, PIC uses as unit of analysis the weighted differences between the phenotypes of sister nodes in the phylogeny (either directly observed in terminal taxa or locally reconstructed from the phenotypes of descendants for internal nodes) and therefore explicitly focuses on evolutionary change. In contrast, in other approaches,

such as phylogenetic generalized least squares, the units of the analysis are the observed taxa (Klingenberg and Marugán-Lobón, 2013). So we chose PIC in this study because the appropriate target of our analyses was the evolutionary change and not the states of taxa.

PIC has been adopted in different study systems under the approach of modularity and morphological integration, such as insect wings (Klingenberg et al. 2001), hominoid cranium (Mitteroecker and Bookstein, 2008), skull in domestic dogs (Drake and Klingenberg, 2010), birds (Klingenberg and Marugán-Lobón, 2013), skulls of salamanders (Adams and Felice, 2014), and the brain of primates (Gómez-Robles et al. 2014).

The resulting phylogeny was used for mapping shape data by squared-change parsimony and to compute PIC (Felsenstein, 1985) to take into account the non-independence of data points due to phylogeny (Klingenberg and Gidaszewski, 2010). All branch lengths were set to the same length (assuming an evolutionary model with the same expected amount of morphological change on every branch). This means that we used unweighted squared-change parsimony (Maddison, 1991) and its equivalent for independent contrasts (Klingenberg and Marugán-Lobón, 2013).

6.2.5. Allometry, evolutionary allometry, and size correction

Since allometry is a factor that can contribute to the integration of the entire morphological structure, we evaluated the allometric effects by performing a multivariate regression of Procrustes coordinates of the ventral and dorsal anchors on lnCS. Allometry tests were based on 10,000 iterations under the null hypothesis of independence between size and shape. The covariance matrices of residuals from these multivariate regressions were used to eliminate the allometric component of shape variation in the analyses of modularity and integration of anchors in *Ligophorus* spp., when the hypotheses were not significant, after removing the influence of allometry (size corrected; Klingenberg, 2009). In an evolutionary context, allometry refers to the multivariate regression of independent contrasts of Procrustes coordinates, as the shape variables, on

independent contrasts of lnCS, as the size measure (Klingenberg, 2013). Because the ordering of sister nodes in the tree is arbitrary, it is necessary to use a regression through the origin for the analysis of independent contrasts (Rohlf, 2001). The residuals resulting from this regression were used to correct the effect of size in the evolutionary modularity and integration tests.

To eliminate the effect of evolutionary allometry, a size correction based on the regression of independent contrasts was applied to the averages of each species of *Ligophorus*. To do this, the vector of regression coefficients computed from independent contrasts was used to decompose the deviations of species mean shapes from the grand mean into predicted and residual components, in a similar way to the phylogenetic size correction described by Revell (2009).

6.2.6. Morphological and evolutionary modularity

We aimed to determine whether the variation in anchor shape is modular. To address this question, four a priori anchor modular hypotheses were designated based on the applied forces exerted by associated muscular systems on the anchors and the functional principles of attachment in *Ligophorus* spp. (Petrov et al. 2015).

H1 (roots/point hypothesis, landmarks 1–4 and 5–8 respectively; Figure 6.1a): The roots are bases for muscle attachment. Biomechanically, the force exerted through muscles and transmitted to the point compartment controls the anchor grip strength on the gills (Khang et al. 2016). Research carried out on *Ligophorus* has shown the shape diversity of ventral and dorsal anchors according the differences in root and point shapes among species (Sarabeev et al. 2013). Thus, we assumed that H1 tests this observed variation within the genus.

H2 (closing/opening hypothesis, landmarks 1, 2, 4, and 6 and 3, 5, 7, and 8, respectively; Figure 6.1b) was proposed based on a previous study of the haptor musculature of *Ligophorus* by Petrov et al. (2015). Extrinsic muscles arising from the inner roots of the anchors, exerting force on

landmarks 1, 2, 4, and 6, close the anchor, whereas muscles running from the outer roots of the anchors to the body wall exerting force on landmarks 3, 5, 7, and 8 open it.

H3 (medial/lateral part hypothesis, landmarks 1, 6, 7, and 8 and 2–5, respectively; Figure 6.1c): We tested a longitudinal partition between the inner root point, middle blade, external curve point and tip point (external part of anchor), and the outer root point, groove point, dent point, internal curve point (internal part of anchor). This hypothesis was defined based on the disposition of muscles connected with the mid-portion of the ventral bar wing-shaped of the ventral bars, which are responsible for the rotation of ventral anchors, and the forces exerted on the medial and lateral parts of the anchor to deepen the point into the gill tissue.

H4 (dorsal/ventral anchors pair hypothesis, landmarks 1–8 of the dorsal anchors vs. landmarks 1–8 of the ventral anchors; Figure 6.1d): Given the differences in shape variation and the potential roles in attachment to the gills between the ventral and dorsal anchors reported in *L. cephali* (Rodríguez- González et al. 2015; Llopis-Belenguer et al. 2015), we tested whether each pair of ventral and dorsal anchors represent two separate modules.

To assess the level of modularity, we quantified the strength of covariation between subsets of landmarks defined by each hypothesis using the *RV* coefficient (Robert and Escoufier, 1976). This coefficient can be interpreted as a multivariate generalization of the bivariate R^2 value (Klingenberg, 2009) on superimposed anchors in the tangent space. To determine whether there is evidence for modularity in the anchors of *Ligophorus* spp., we compared the *RV* coefficient with those obtained from alternative random partitions of the configuration into subsets of landmarks. The subsets had the same number of landmarks as the hypothesized modules, and alternative partitions were either spatially contiguous (testing of H1 and H3) or non-contiguous (testing of H2 and H4; Klingenberg, 2009). Spatial contiguity was defined using an adjacency graph based on Delaunay triangles to outline the potential partitions of the anchors used during randomization

(Klingenberg, 2009). Lower values of the observed *RV* coefficient in the left tail of the distribution would indicate a significant partition of the two subsets of landmarks (Klingenberg, 2011).

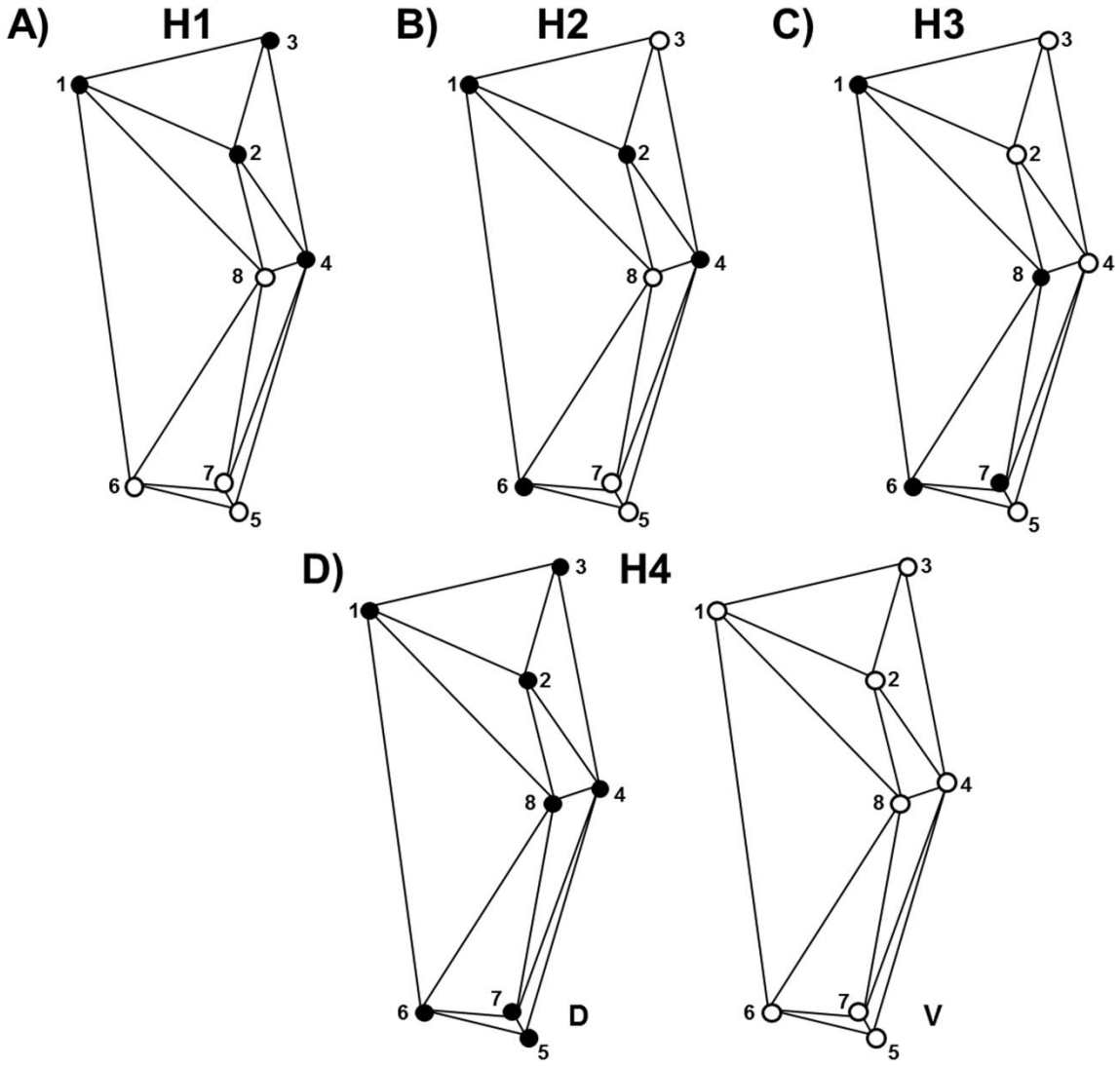

Figure 6.1. Morphological modularity of the hypothesized partition schemes on the ventral and dorsal anchors of *Ligophorus* spp. A) Partition of the landmarks into subsets corresponding to hypothesis 1 (H1), roots (filled circles), and points (unfilled circles). B) H2: opening (unfilled circles) and closing (filled circles) in the anchors. C) H3: medial (filled circles) and lateral (unfilled circles) parts of the inside of anchors. D) H4, where the whole dorsal (D) and ventral (V) anchors act as a unit of attachment.

We also applied the test of modularity at the level of evolutionary variation using the covariance matrix of PIC (Felsenstein, 1985; Drake and Klingenberg, 2010). As in the preceding analyses, this test uses the uncentered covariance matrix of independent contrasts to take into account the arbitrary ordering of sister nodes from which the contrasts are computed.

6.2.7. Morphological and evolutionary integration

In case of significant modularity, the nature of covariation between modules was investigated with a two-block partial least squares (PLS) analysis (Rohlf and Corti, 2000). PLS analysis provides new shape variables that maximize the covariance among landmark configurations of the different modules and therefore can be interpreted as the main feature of integration among them (Klingenberg, 2014). To assess the observed singular-value decomposition and correlations, 10,000 permutation tests were performed. A multivariate regression between the PLS blocks on the first axis and the corresponding Procrustes coordinates in a tangent space was performed to visualize shape changes into the first pair of the PLS axis (Bookstein et al. 2003).

Likewise, to assess the covariation of evolutionary changes of the significant modularity hypotheses, we used PLS analysis with independent contrasts of the shape variables (Klingenberg and Marugán-Lobón, 2013). Because independent contrasts represent evolutionary change, the covariation between independent contrasts of the shape coordinates for partitioned anchor modules indicates evolutionary integration of shape between them. Therefore, the PLS axes calculated from independent contrasts identify the shape features with maximal evolutionary covariation (Klingenberg and Marugán-Lobón, 2013). In this case, we computed the singular-value decomposition from an uncentered covariance matrix so that the results are unaffected by the arbitrary ordering of sister nodes from which the contrasts are obtained (Klingenberg and Marugán-Lobón, 2013). These statistical analyses were carried out with MorphoJ v. 1.06e (Klingenberg, 2011).

6.3. Results

6.3.1. Evolutionary allometry

The multivariate regression indicated a significant relationship ($P < 0.0001$) between shape and lnCS based on species means of *Ligophorus* in ventral and dorsal anchors. However, this relationship accounted only for a modest portion (9.18 and 3.29 % in the ventral and dorsal anchors, respectively) of the total shape variation (Figures 6.2A and 6.3A).

The same analysis based on independent contrasts yielded no evidence for allometry between shape and lnCS of the ventral and dorsal anchors ($P > 0.25$; Figures 6.2B and 6.3B). The allometry plots showed short and elongated shapes for ventral anchors and short and narrow shapes for dorsal anchors (Figures 6.2B and 6.3B).

6.3.2. Modularity, size-corrected, and evolutionary modularity

The modularity hypothesis H1 was supported by the observed *RV* coefficients for the ventral and dorsal anchors, being significantly lower than those expected by chance at simple level (*RV*= 0.623, *P*= 0.0486 and *RV*= 0.288, *P*= 0.0035, respectively; Figures 6S2A, D). In contrast, the *RV* coefficients computed under H2 and H3 for both ventral and dorsal anchors did not support a modular arrangement ($P > 0.05$; Figure 6S2B, C, E, F). In addition, the value of *RV* testing H4 supported the notion of the ventral and dorsal anchors representing two modules (*RV*= 0.670, *P*= 0.047; Figure 6S3). The size-corrected contrast shape was applied on modularity tests and was not significant ($P > 0.05$) for H2 and H3 in the ventral and dorsal anchors (Table 6S1).

The analysis of evolutionary covariation between the hypothesized ventral anchor modules to the covariation in the species of *Ligophorus* did not support H1 and H2 ($P > 0.05$; Figure 6S4A, B). H3 was significant for ventral anchors (*RV*= 0.663, *P*= 0.015; Figure 6S4C), but not for dorsal ones ($P > 0.05$; Figure 6S4D–F); H4 was also significant (*RV*= 0.697, *P*= 0.026; Figure 6S5). The same pattern

of statistical support was observed when the allometric size correction on evolutionary modularity was applied (Table 6S2).

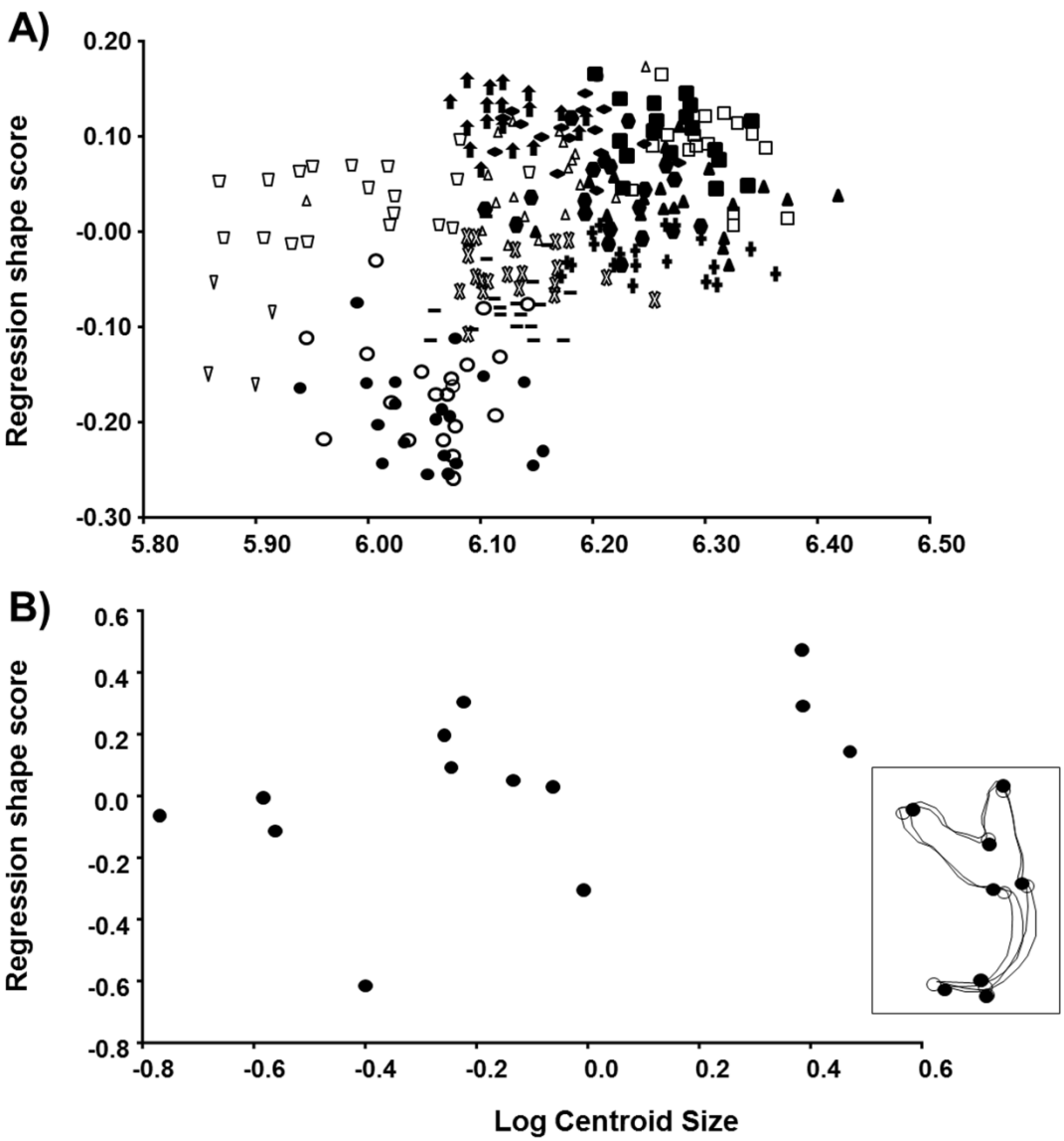

Figure 6.2. Allometry in ventral anchors of *Ligophorus* spp. A) *Ligophorus* species' average shapes regressed on log-transformed centroid size reveal a significant relationship ($P < 0.001$) accounting for 9.18 % of the total shape variation. *Ligophorus acuminatus* (*filled square*), *Ligophorus imitans* (◂), *Ligophorus confusus* (*filled circle*), *Ligophorus vanbenedenii* (▽), *Ligophorus szidati* (*unfilled circle*), *Ligophorus cephali* (_), *Ligophorus chabaudi* (⧖), *Ligophorus heteronchus* (*upward arrow*), *Ligophorus minimus* (*unfilled square*), *Ligophorus mediterraneus* (*filled cross*), *Ligophorus macrocolpos* (*unfilled triangle*), *Ligophorus pilengas* (*filled triangle*), *Ligophorus angustus* (*unfilled inverted triangle*), and *Ligophorus llewellyni* (⬣). B) Evolutionary allometry. Regression of phylogenetic independent contrasts of shape and log-transformed centroid size reveals no significant relationship ($P > 0.05$) accounting for 8.37 % of the total shape variation.

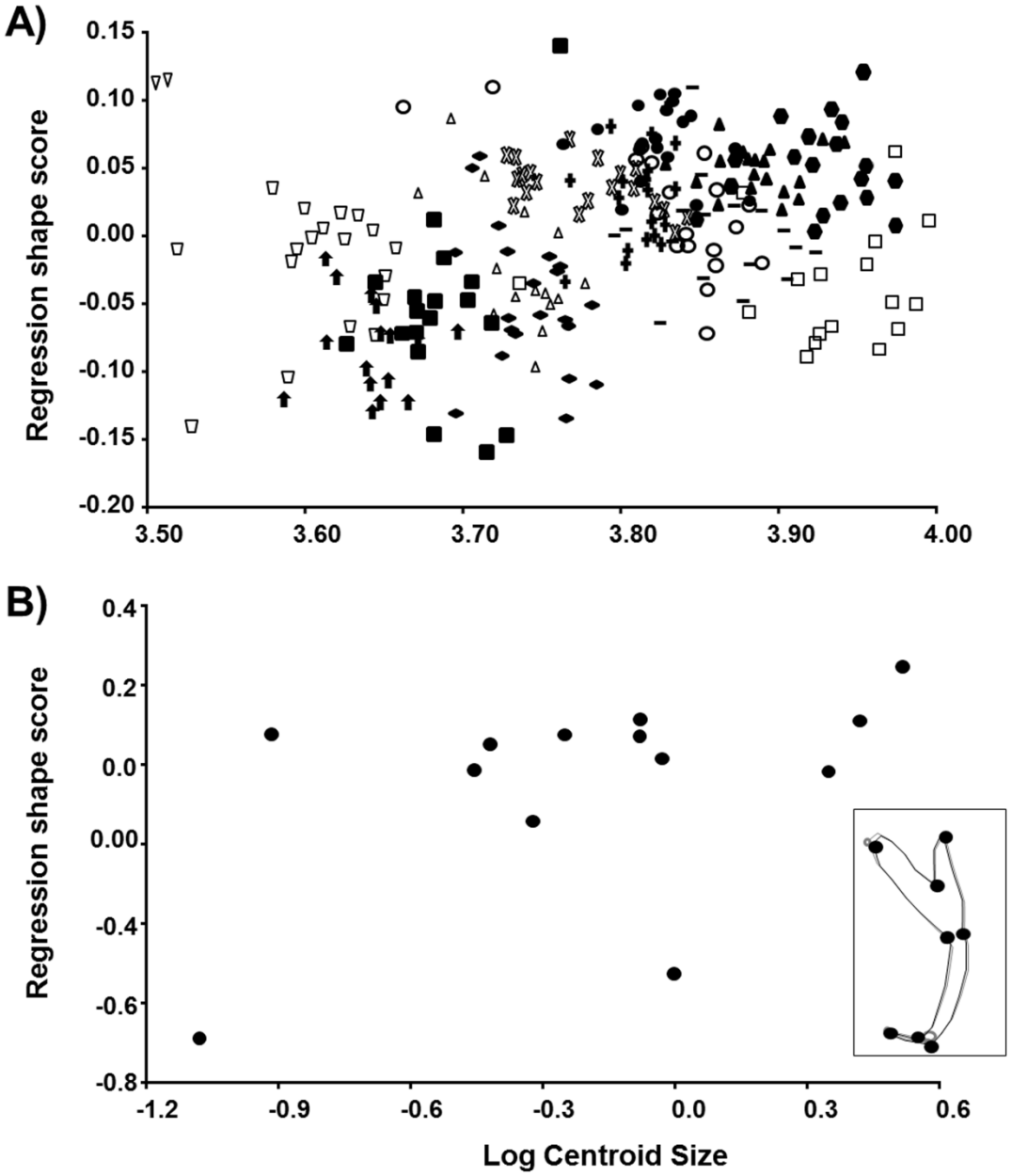

Figure 6.3. Allometry in dorsal anchors of *Ligophorus* spp. A) *Ligophorus* species' average shapes regressed on log-transformed centroid size reveal a significant relationship ($P < 0.001$) accounting for 3.29 % of the total shape variation. Symbol descriptions as in Figure 6.2. B) Evolutionary allometry. Regression of phylogenetic independent contrasts of shape and log-transformed centroid size reveals no significant relationship ($P > 0.05$) accounting for 10.01 % of the total shape variation.

6.3.3. Morphological and evolutionary integration

Covariation between the modules of H1 of the ventral and dorsal anchors was highly significant ($P < 0.001$; Figures 6.4 and 6.5). The first two pairs of the PLS axes accounted for 96.1 and 2.9 % of the total covariance for ventral and 75.9 and 14.1 % for dorsal anchors. The scatter plots using the first pair of PLS axes indicated a strong covariation between the modules. The high scores on PLS axis 1 show short shapes of ventral anchors and points and an elongated and narrow shape for dorsal anchors, as displayed by the warped outline drawing (Figures 6.4 and 6.5). Integration between pairs of anchors (H4) was also significant ($P < 0.001$; Figure 6.6). The warped outlines pointed to more variation in the anchor roots and points (see figures of anchors inside the plots).

PIC analysis indicated strong evolutionary integration between the roots and points in the ventral and dorsal anchors (H1; Figure 6.7a, b), between the medial and lateral modules (H3) only for ventral anchors (Figure 6.7c), and between ventral and dorsal anchors (H4; Figure 6.7d).

6.4. Discussion

This study used geometric morphometrics in a comparative context to investigate evolutionary modularity and morphological integration in the haptoral anchors across species of *Ligophorus*. This tool is well suited for studying these two mechanisms by configurations of landmarks that can be decomposed into sensible units. Our main purposes were to address whether the anchors act as a single integrated unit or whether they represent several distinct modules and, if so, whether they evolve as distinct units. Our analyses showed that there was strong integration of evolutionary changes throughout the anchors.

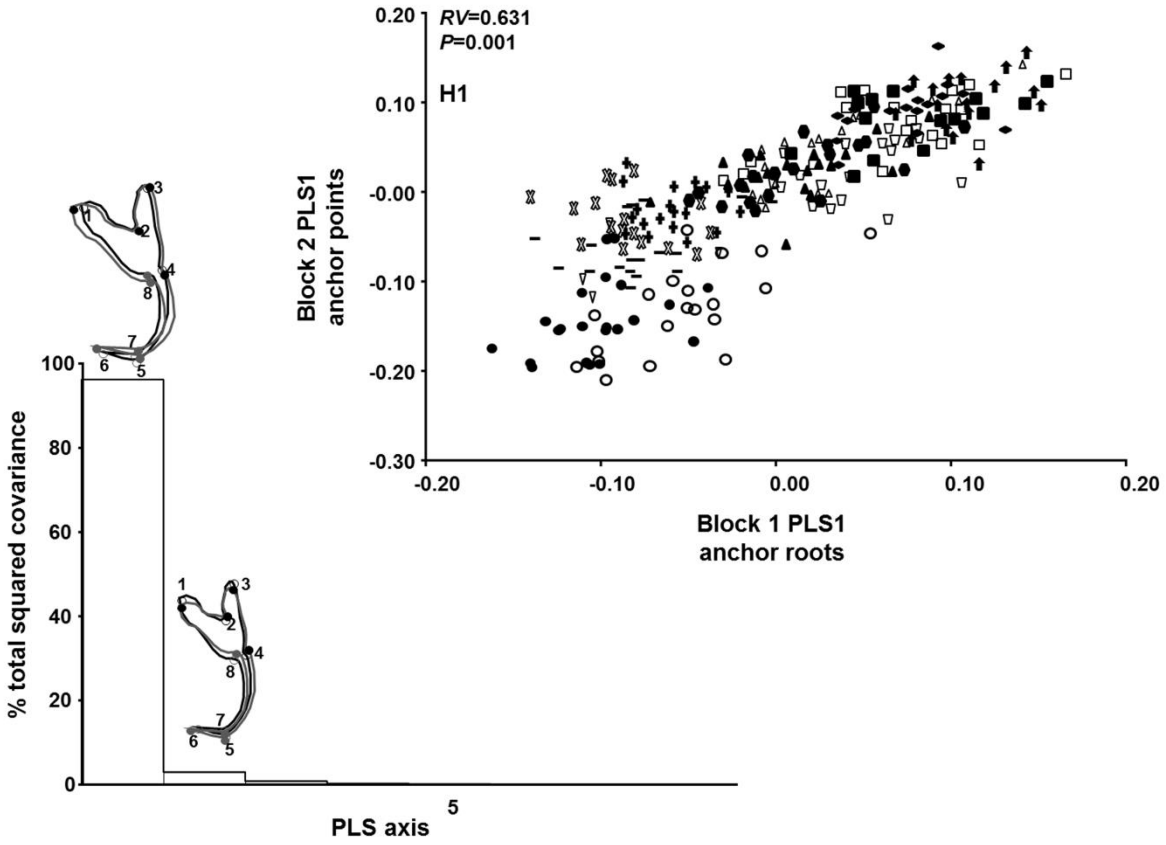

Figure 6.4. Morphological integration in ventral anchors (*H1*). a) Plot of scores of the first partial least square (*PLS1*) illustrating the pattern of maximum covariation between the roots and points of the ventral anchors in 14 species of *Ligophorus*. The percentage of the total of covariance and the warped outline drawing display the shape variation in correspondence to the extreme values of each axis for the two first PLS. *Scale factor*, 0.1. Symbol descriptions as in Figure 6.2.

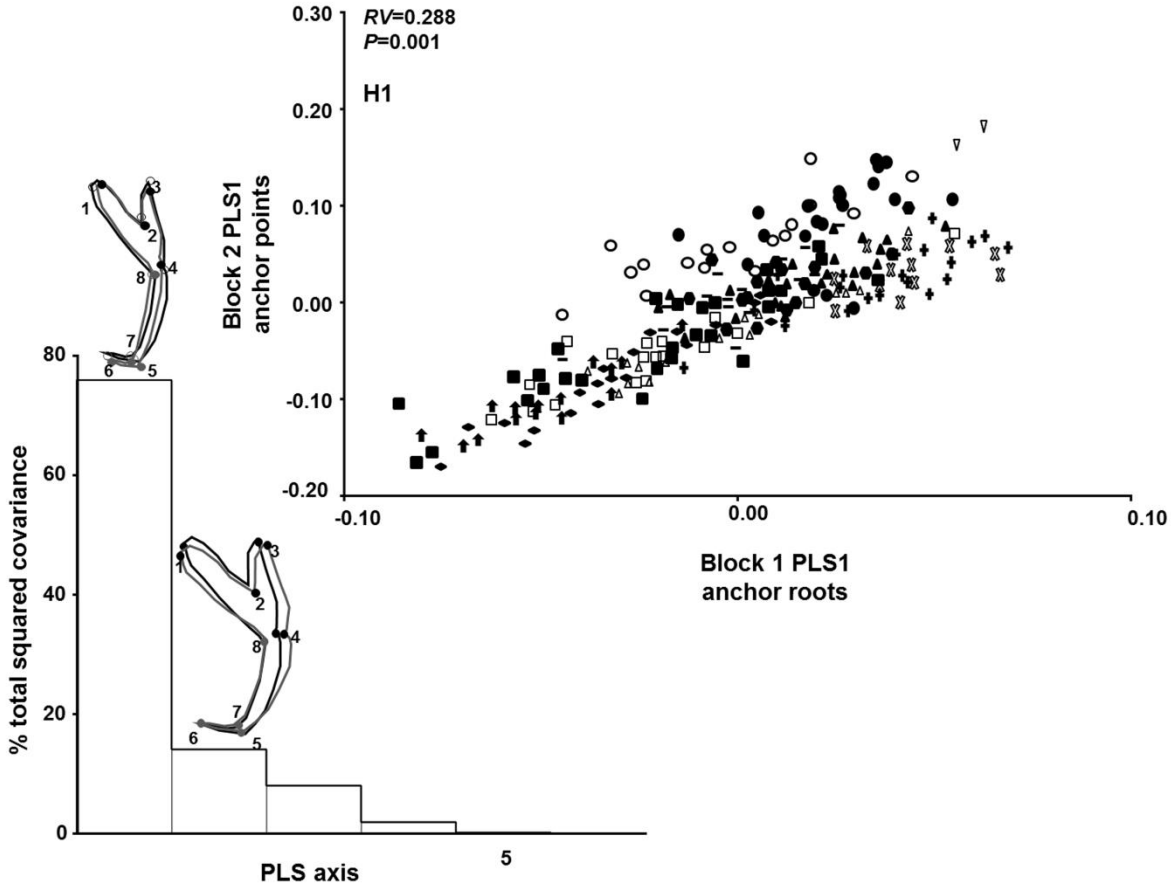

Figure 6.5. Morphological integration in dorsal anchors (*H1*). a) Plot of scores of the first partial least square (*PLS1*) illustrating the pattern of maximum covariation between the roots and points of the ventral anchors in 14 species of *Ligophorus*. The percentage of the total of covariance and the warped outline drawing display the shape variation in correspondence to the extreme values of each axis for the two first PLS. *Scale factor*, 0.1. Symbol descriptions as in Figure 6.2.

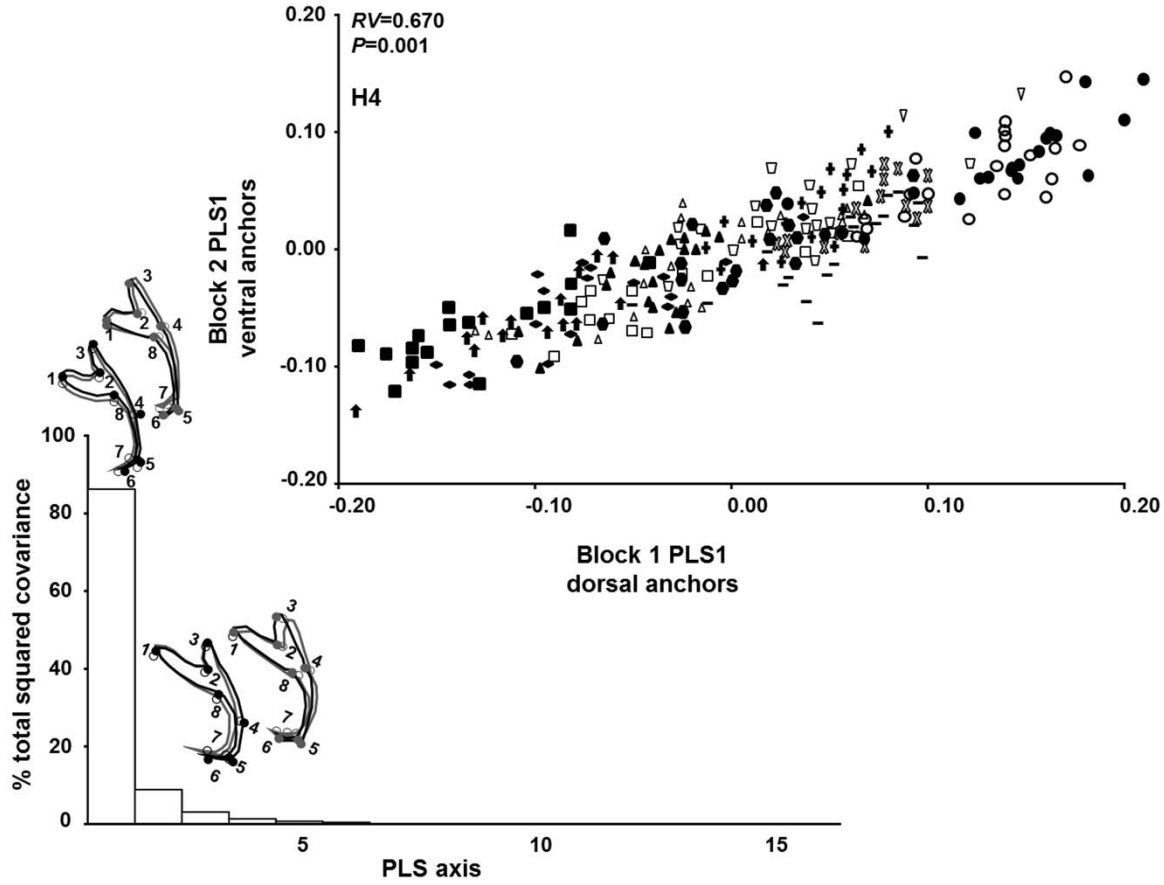

Figure 6.6. Morphological integration in the ventral-dorsal anchor (*H4*). a) Plot of scores of the first partial least square (*PLS1*) illustrating the pattern of maximum covariation between the roots and points of the ventral anchors in 14 species of *Ligophorus*. The percentage of the total of covariance and the warped outline drawing display the shape variation in correspondence to the extreme values of each axis for the two first PLS. *Scale factor*, 0.1. Symbol descriptions as in Figure 6.2.

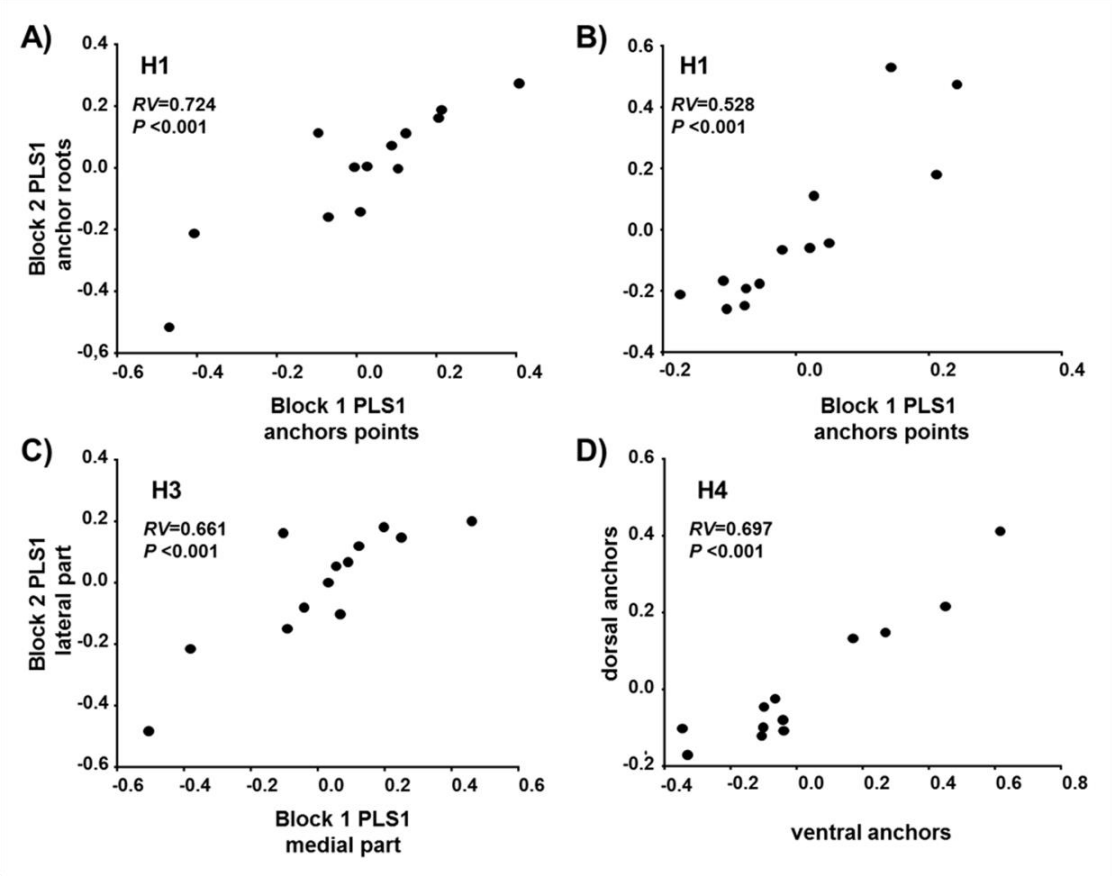

Figure 6.7. Evolutionary morphological integration. Partial least squares analysis from phylogenetic independent contrasts. A) Evolutionary integration between root and point ventral anchors. B) Evolutionary integration between root and point dorsal anchors. C) Evolutionary integration between the medial and lateral parts of ventral anchors. D) Evolutionary integration in the ventral–dorsal anchors.

The results of evolutionary integration indicated that ventral and dorsal anchors appear to evolve as single integrated units for attachment to the gills of host, which means that anchors can be considered as a single, fully integrated coherent structure. Here, we evaluate these findings.

6.4.1. Allometry and evolutionary allometry

The effect of size on shape may produce global integration throughout the whole morphological structure, counteracting modularity (Jojić et al. 2012). However, although allometry was a contributing factor in our modularity assessment, it only accounted for a small portion of the

anchor shape variation, and even its influence was statistically non-significant when parasite phylogeny was taken into account.

6.4.2. Morphological and evolutionary modularity

We have provided evidence that the variation in the anchors of *Ligophorus* spp. is concentrated in some modules. The anchors exhibited a significant level of covariation between two compartments of attachment: roots and points (H1) for ventral and for dorsal anchors, being moderate for ventral and low level of covariation for dorsal anchors. The dorsal–ventral pair of anchor shapes (H4) showed significant moderate level of covariation in the species of *Ligophorus*, suggesting that ventral and dorsal anchors represent two independent modules. These results may suggest that attachment to the gills is composed of two subunits. The occurrence of modules in H1 in the ventral and dorsal anchors was not corroborated when parasite phylogeny was accounted for, which may be indicative of convergent evolution (similar anchor morphologies in relatively unrelated species as a response to similar ecological/functional constraints). Homoplasy can be expected in the morphology of attachment organs in parasites because functional requirements for attaching to the host and adapting to within-host microhabitats would counterbalance shape constraints imposed by phylogeny (Khang et al. 2016). In fact, high levels of homoplasy in the haptor have been observed in species of *Ligophorus* (Sarabeev and Desdevises, 2014).

The putative diversity and complexity of microhabitats (gill arch, segment, or area) provided by fish gills are continually exposed to strong gill ventilating currents and may exert strong selective pressures, resulting in adaptation to particular gill areas (Šimková et al. 2004; Justine et al. 2013; Kearn, 2014). In *Dactylogyrus*, species occupying similar niches tend to have similar morphology of attachment organs (Šimková et al. 2002). These adaptive responses could have facilitated the formation of modules in H1 for the ventral and dorsal anchors of species of *Ligophorus*. The

significant relationship between host and parasite size seems to indicate an adaptation of the parasite to mechanical constrains in the gill chamber (Sasal et al. 1999).

The tests of H4 clearly demonstrated a bipartition of variation in the ventral and dorsal anchors forming two modules, which points to each type of anchor having different functional roles for attachment to the gills. Similar results were observed in the attachment organs of *Cichlidogyrus* spp., where three main morphological configurations, which included the ventral and dorsal anchors as two relatively independent modules, were proposed (Vignon et al. 2011). These authors found no clear relationship between host specificity and morphological modularity, and geographical distribution (Vignon et al. 2011). However, Messu Mandeng et al. (2015), considering a more distant host switch, provided evidence supporting the hypothesis of the adaptive nature of haptor morphology within *Cichlidogyrus*. In *L. cephali*, Rodríguez-González et al. (2015) showed that shape variation is much larger in dorsal than in ventral anchors and proposed that it could indicate different roles in attachment. In addition, Llopis-Belenguer et al. (2015) found differences in the strength on phenotypic buffering variation of the ventral and dorsal anchors in *L. cephali*. All this evidence agrees with the notion of two separate modules in *Ligophorus* spp. presented herein, and the shape covariation detected indicates that the dorsal and ventral anchors form two relatively independent evolutionary modules.

In this evolutionary scenario, the arrangement defined in H3 (Figure 6S4C) showed significant congruence with PIC in the ventral anchors. This revealed phylogeny to be a major determinant of ventral anchor shape variation, in accordance with other monogeneans such as *Cichlidogyrus* (Vignon et al. 2011; Khang et al. 2016). The moderate levels of anchor shape–size covariation suggest that apart from the effect of shared ancestry, anchor shape–size covariation is likely non-trivially constrained by additional factors, one of which could be their mechanical compatibility.

The important role of anchor muscles in attachment has been discussed in different monogeneans (Petrov et al. 2015, 2016). These patterns of variability in the muscles in different

species could also explain the different patterns of modularity in relation to a specific mode of attachment and requirements of its host.

In *Ligophorus* spp., the arrangement of the haptoral musculature has been shown to be identical to *L. pilengas* and *L. llewellyni* and similar to *Ligophorus kaohsianghsieni*, Gusev (1985) (Petrov et al. 2015). This architecture of haptoral musculature may be exerting similar forces in the species of *Ligophorus* studied here, principally in the roots (inner and outer) and points, forming functional modules for attachment to gills.

The PIC analyses did not allow identifying modules in the anchors within species of *Ligophorus* (Figure 6S4A, B, D–F) for ventral and dorsal anchors, respectively. These results may explain divergence by increasing in the adaptability to different gill microhabitats (Khang et al. 2016).

6.4.3. Morphological and evolutionary integration

The results of morphological integration between modules found in the anchors of *Ligophorus* spp. revealed that the covariation between roots and points, and medial and lateral anchor shapes was highly significant for ventral anchors and low for dorsal anchors. It is thus plausible that the compartments of anchors in *Ligophorus* spp. are well integrated. Such integration may be a product of common inheritance due to pleiotropy or linkage disequilibrium or the result of the concerted evolution of morphological elements that operate together to perform a specific function (Sánchez and Lasker, 2003). Although the proximate cause is not well known, this probably arises because the anchor components evolve, develop, and operate jointly in a coordinated manner.

Recently, Rodríguez-González et al. (2015) showed similar results at intraspecific morphological integration in the same structure (root and point anchors) in *L. cephali*, indicating a concerted action between them, which is related to H1. Similar results were observed by Khang et

al. (2016) when extending their morphological integration to the interspecific level in 13 species of *Ligophorus* off West Peninsular Malaysia between roots and points in ventral and dorsal anchors.

Our analysis based on comparative methods showed that there is strong integration of evolutionary changes throughout the anchors, specifically in H1, H3 (only for ventral anchors), and H4. Allometry was a contributing factor to this integration, but does not account for all integration in the anchors. The evolutionary integration may be related to the arrangements of the muscles of species of *Ligophorus*, as described by Petrov et al. (2015), showing a degree of complexity sufficient to effect a set of highly coordinated and precise movements of anchors and connecting bars.

Therefore, the morphological integration in the species of *Ligophorus* evaluated by PIC was a major determinant in the morphological integration than modularity, which is in line with previous studies of *Ligophorus* spp. from *Liza subviridis* and *Moolgarda buchanani* (Khang et al. 2016), and also has been demonstrated to be a determinant important in the anchor shape variation in *Cichlidogyrus* (Vignon et al. 2011). Consequently, the formation of modules in the anchors can be considered a trend in monogeneans as organismal integration and complexity increases.

Independent contrasts are a convenient way to use tools of geometric morphometrics in the context of phylogenetic comparative approaches (Klingenberg and Marugán-Lobón, 2013) with a statistical power. These may apply at multiple levels of variation, such as fluctuating asymmetry within individuals, phenotypic and genetic variation among individuals within taxa, and evolutionary variation among taxa. We believe that the adoption of such multilevel analyses would allow unprecedented inferences on the evolutionary processes in Monogenea.

In this study, we have demonstrated that, apparently, there are two processes (adaptive–evolutionary) that trigger the formation of modular H1, H3, and H4 and showed evidence for a significant interspecific morphological integration of these modules. To advance our understanding of variability of attachment organ in monogeneans, parasite phylogeny needs to be explicitly linked with anchor morphometry.

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Compliance with ethical standards

Conflicts of interest: The authors declare that they have no conflict of interest.

Statement of human rights: For this type of study, formal consent is not required.

Statement on the welfare of animals: All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Supplementary material

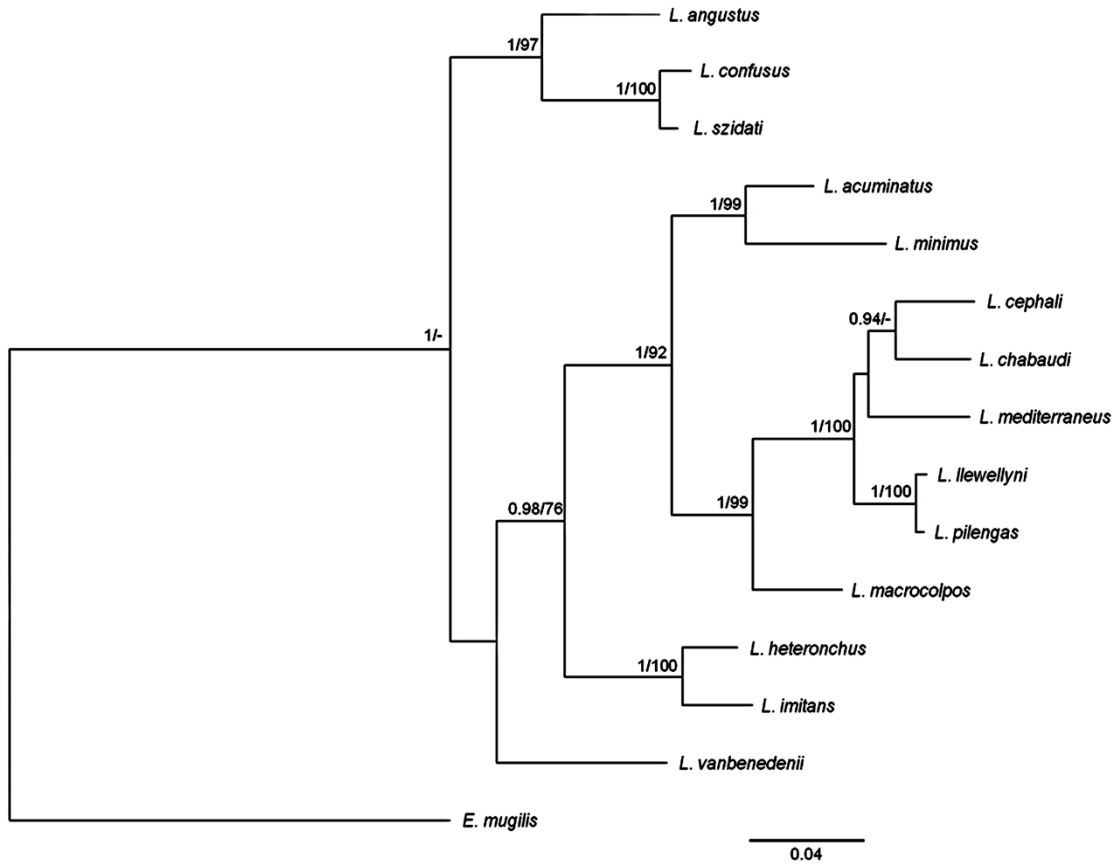

Figure 6S1 Phylogentic tree of *Ligophrous* spp. from the Mediterranean and Black Sea derived from Bayesian Inference and Maximum Likelihood analysis using 28S and ITS1 regions. Posterior probability values are indicated above the branches, followed by maximum likelihood bootstrap values (in %). Posterior probabilities <0.80 and bootstrap values <60% not reported.

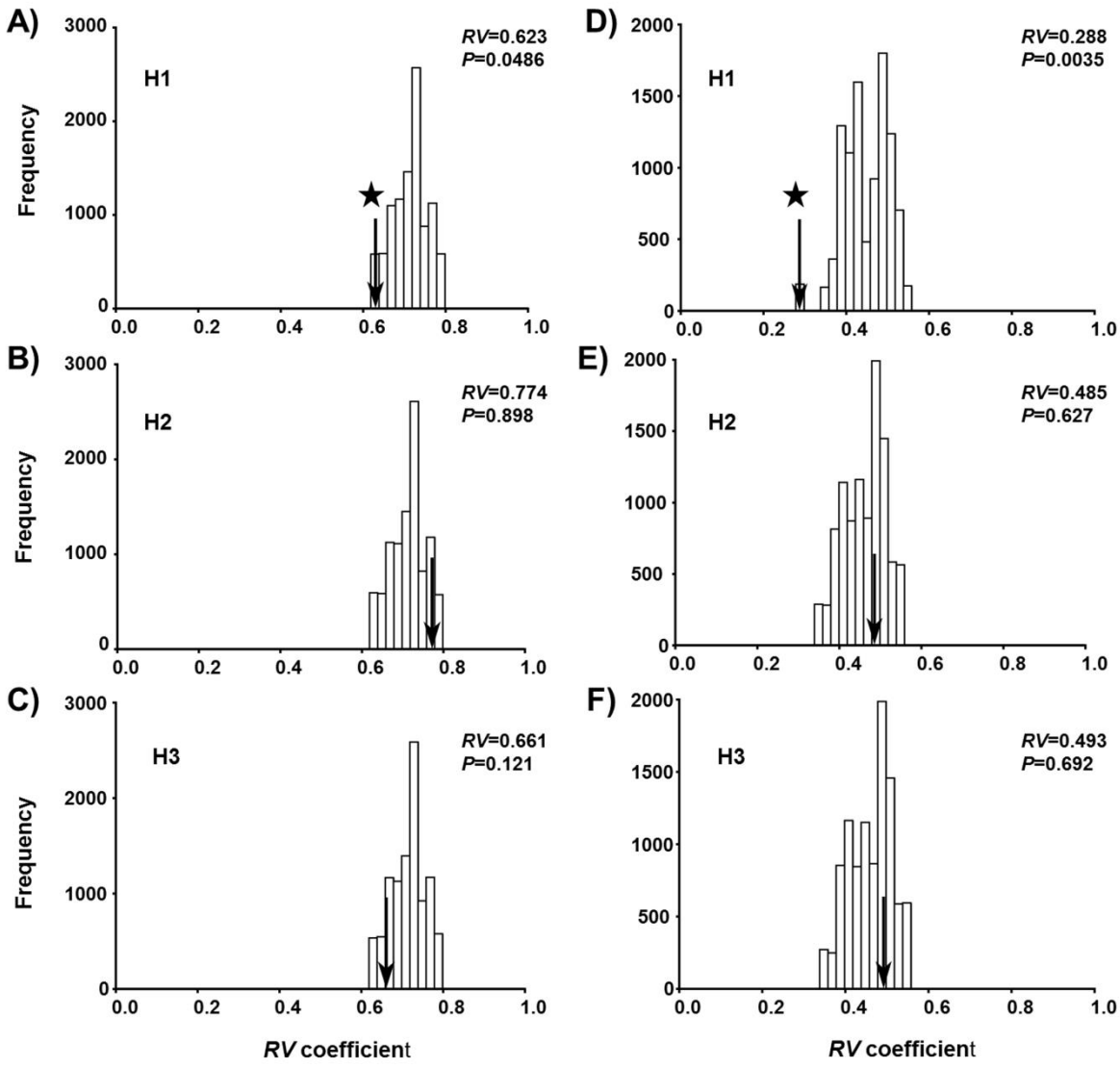

Figure 6S2 Analysis of modularity in the ventral and dorsal anchors. Graphs show the *RV* coefficients for the subdivision of landmarks into anchors and the distribution of *RV* coefficients, for 10,000 alternative partitions of landmarks into anatomically contiguous (H1 and H3 hypotheses) and non-contiguous (H2) subsets (histograms). A-C) represents ventral anchors, and D-F) represents dorsal anchors. Black arrows indicate the observed *RV* coefficients value and black stars indicate of significant partitions.

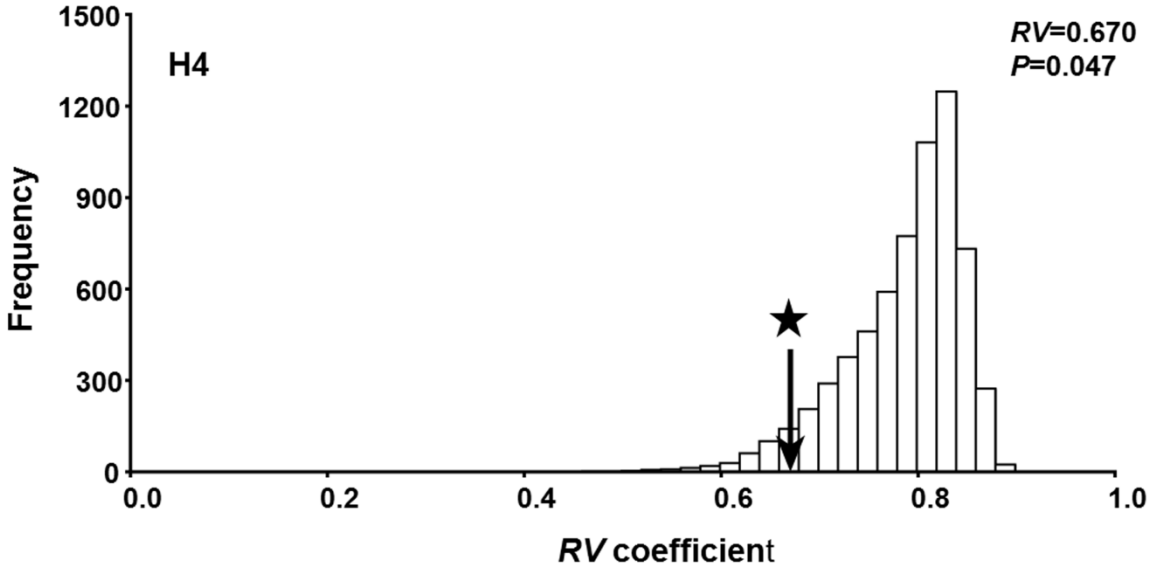

Figure 6S3 Analysis of modularity in the ventral-dorsal anchors (H4). Graphs show the *RV* coefficients for the subdivision of landmarks in separate anchors and the distribution of *RV* coefficients, for 10,000 alternative partitions of landmarks in anatomically non-contiguous subsets (histogram). Black arrows indicate the observed *RV* coefficients value and black stars indicate of significant partitions.

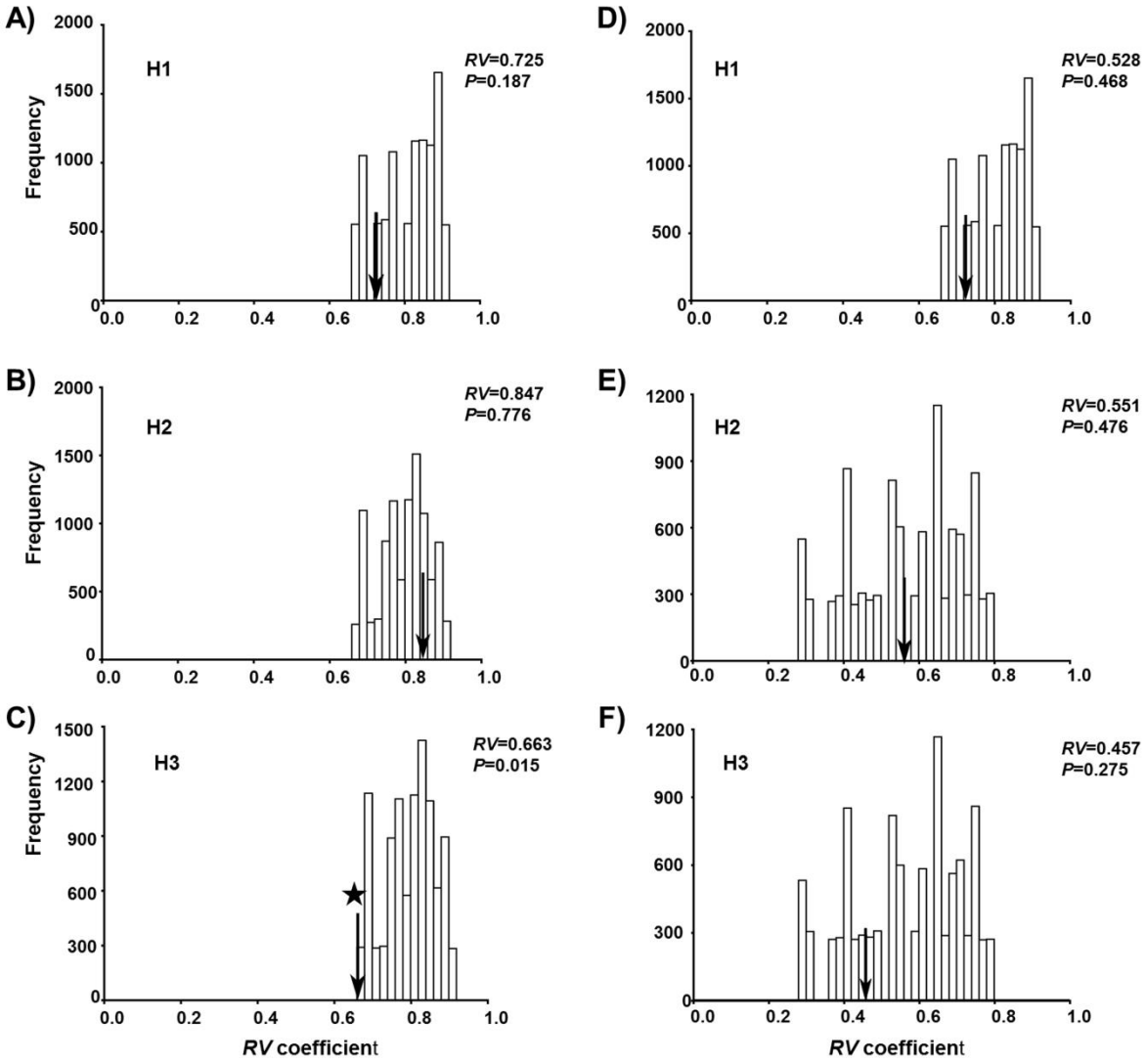

Figure 6S4 Evolutionary analysis of modularity in ventral and dorsal anchors with Phylogenetic independent contrasts (PIC). Graphs show the *RV* coefficients for the subdivision of landmarks into anchors and the distribution of *RV* coefficients, for 10,000 alternative partitions of landmarks into anatomically contiguous H1 and H3 and non-contiguous H2 hypotheses. A-C) represents ventral anchors and D-F) represents the dorsal anchors. Black arrows indicate the observed *RV* coefficients value and black stars indicate of significant partitions.

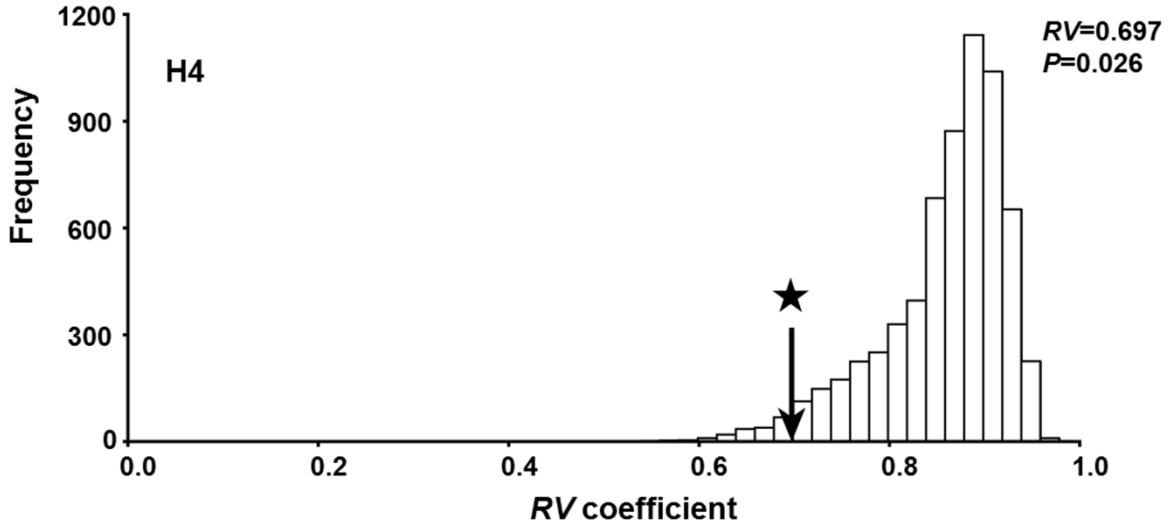

Figure 6S5 Evolutionary analysis of modularity in the ventral-dorsal anchors (H4). Graphs show the *RV* coefficients for the subdivision of landmarks in separate anchors and the distribution of *RV* coefficients, for 10,000 alternative partitions of landmarks in anatomically non-contiguous subsets (histogram). Black arrows indicate the observed *RV* coefficients value and black stars indicate of significant partitions.

Supplementary material

Table 6S1. Size-corrected on modularity hypotheses.

Hypotheses	Ventral anchors	Dorsal anchors
H1	*	*
H2	*RV*= 0.740, *P*= 0.908	*RV*= 0.480, *P*= 0.644
H3	*RV*= 0.636, *P*= 0.094	*RV*= 0.491, *P*= 0.730

Table 6S2. Size-corrected on evolutionary modularity hypotheses.

Hypotheses	Ventral anchors	Dorsal anchors
H1	*RV*= 0.674, *P*= 0.182	*RV*= 0.588, *P*= 0.413
H2	*RV*= 0.871, *P*= 0.798	*RV*= 0.590, *P*= 0.392
H3	*	*RV*= 0.557, *P*= 0.297

* Significant hypotheses

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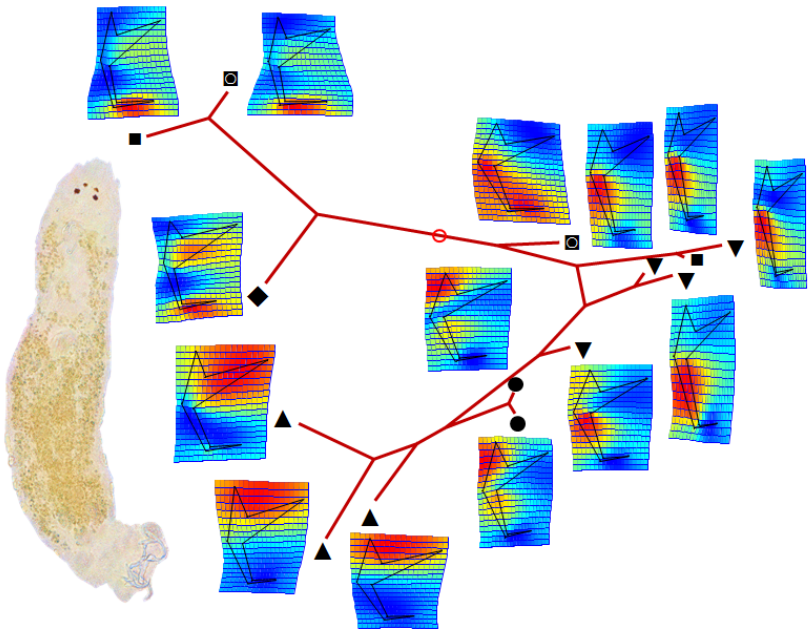
CHAPTER 7

EVOLUTIONARY MORPHOLOGY IN SHAPE AND SIZE OF HAPTORAL ANCHORS IN 14 *LIGOPHORUS* SPP. (MONOGENEA: DACTYLOGYRIDAE)



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Abstract

The search for phylogenetic signal in morphological traits using geometric morphometrics represents a powerful approach to estimate the relative weights of convergence and shared evolutionary history in shaping organismal form. We assessed phylogenetic signal in the form of ventral and dorsal haptoral anchors of 14 species of *Ligophorus* occurring on grey mullets (Osteichthyes: Mugilidae) from the Mediterranean, the Black Sea and the Sea of Azov. The phylogenetic relationships among these species were mapped onto the morphospaces of shape and size of dorsal and ventral anchors and two different tests were applied to establish whether the spatial positions in the morphospace were dictated by chance. Overall significant phylogenetic signal was found in the data. Allometric effects on anchor shape were moderate or non-significant in the case of evolutionary allometry. Unrelated species occurring on the same host differed markedly in anchor morphology indicating little influence of host species on anchor form. Our results suggest that common descent and shared evolutionary history play a major role in determining the shape and, to a lesser degree in the size of haptoral anchors in *Ligophorus* spp. The present approach allowed tracing paths of morphological evolution in anchor shape. Species with narrow anchors and long shafts were associated predominately with *Liza saliens*. This morphology was considered to be ancestral relative to anchors of species occurring on *Liza haematocheila* and *M. cephalus* possessing shorter shafts and longer roots. Evidence for phylogenetic signal was more compelling for the ventral anchors, than for the dorsal ones, which could reflect different functional roles in attachment to the gills. Although phylogeny and homoplasy may act differently in other monogeneans, the present study delivers a novel framework to address effectively the relationships among morphology, phylogeny and other traits, such as host specificity or niche occupancy.

Keywords: *Ligophorus*, Geometric morphometrics, Phylogenetic signal, Phylomorphospace, Convergence, Evolution.

7.1. Introduction

Darwin's view of species as evolving entities only detectable by gaps in morphological variation (Mallet, 2010) established an explicit link between morphology and evolution. This inception has pervaded biological thought until today, to the point that it can be asserted that all post-Darwinian morphology has been, to a greater or lesser extent, evolutionary (Richter and Wirkner, 2014). In comparative morphology, the relationship between morphology and evolution is assessed by identifying homologies and determining the chronological order of transformations of evolutionary units (Richter and Wirkner, 2014). The similarity among forms of different species can be explained by inheritance from a common ancestor or by convergence where the form can arise more than once across taxa in response to similar ecological, adaptive, functional, and/or developmental pressures (Revell, 2014; Armbruster et al. 2016). Both processes act concurrently and disentangling their roles has been until recently a daunting task. However, the current availability of phylogenetic tools, coupled with the development of geometric morphometrics methods that can examine morphological data as independent from the effect of phylogeny have greatly simplified this endeavour (Blomberg et al. 2003; Felsenstein, 2004).

Historically, the tendency for related species to resemble one another more than species drawn at random from the same tree has been termed "phylogenetic signal" (Blomberg et al. 2003; Münkemüller et al. 2012). Hence, determining the degree to which traits exhibit phylogenetic signal is crucial to understand how species vary phenotypically and to infer the evolutionary processes that have shaped their phenotypic diversity over evolutionary time (Adams, 2014). In addition, to allow controlling for the confounding effect of phylogenetic dependence, estimation of phylogenetic signal provides a predictive framework of the value of a given trait for a species or an ensemble of closely related species based on their phylogenetic position (Blomberg et al. 2003). The latter is important for parasites because their small size and cryptic natural history hampers the estimation of phenotypic and ecological traits (Krasnov et al. 2011). However, phylogenetic signal

in parasites has rarely been the focus of rigorous analyses (Sasal et al. 1998; Mouillot et al. 2005; Mouillot et al. 2006). Most studies have been chiefly based on comparison of ecological traits, such as abundance and host specificity to investigate diversification, diversity and community ecology (Sasal et al. 1998; Desdevises, 2001; Mouillot et al. 2006; Krasnov et al. 2011; Koehler et al. 2012; Krasnov et al. 2015; Morand et al. 2015), whereas few have considered morphological traits (Vignon et al. 2011; Khang et al. 2016).

Haptoral structures in Monogenea provide an exceptional platform for comparative morphology. On the one hand, and as in any other set of organisms, phylogenetic constraints are expected to account for morphological similarity between species. In fact, haptoral morphology has been found to be suitable for inferring phylogenetic relationships in different monogenean taxa (Pouyaud et al. 2006; Šimková et al. 2006; Vignon et al. 2011; Mendlová et al. 2012; Sarabeev and Desdevises, 2014). On the other hand, the attachment structures of monogeneans are subjected to strong selective pressures. In gill monogeneans, these pressures are exerted by both the structural complexity of fish gills, thereby offering a wide variety of microhabitats, and exposure to mechanical stress generated by ventilating currents (Timi, 2003). In fact, Šimková et al. (2002) posit that the morphology of the haptor is, to a large degree, determined by adaptation to the host (host specificity) and to specific sites within their hosts (niche preference), which has been corroborated in, for instance, *Lamellodiscus* spp. (Poisot et al. 2011). However, other studies indicate that haptor morphology seems to be driven by a combination of both adaptive forces and phylogenetic constraints (Messu Mandeng et al. 2015). For instance, we (Rodríguez-González et al. 2016) showed that different modular arrangements in the anchors of *Ligophorus* spp. could be accounted for by both adaptive and phylogenetic factors acting at different levels.

Ligophorus represents a genus of gill monogeneans exclusive to grey mullets (Osteichthyes: Mugilidae). This host-parasite system has several features that make it invaluable as a model system for studying the evolutionary processes that drive its past diversification and present

diversity (Sarabeev and Desdevises, 2014; Khang et al. 2016). The genus is speciose (some 60 valid species) and morphologically diverse (Sarabeev et al. 2013; Rodríguez-González et al. 2015a). Well-resolved phylogenies are available (Blasco-Costa et al. 2012; Sarabeev and Desdevises, 2014; Khang et al. 2016) and specimens can be easily obtained in large numbers. *Ligophorus* spp. exhibit strict host specificity and several congeneric species tend to occur on the same hosts (Blasco-Costa et al. 2012; Sarabeev et al. 2013; Sarabeev and Desdevises, 2014). Geometric morphometrics has already been applied to *Ligophorus* spp. to explore the correlation between phenotypic variation in attachment organs and factors such as phylogeny, to elucidate mechanisms determining phenotypic buffering, character displacement, as well as in species discrimination (Blasco-Costa et al. 2012; Sarabeev et al. 2013; Llopis-Belenguer et al. 2015; Rodríguez-González et al. 2015b; Khang et al. 2016).

In the present paper, we evaluate the relationship between the form (i.e., the combination of shape and size) (Klingenberg, 2016) of haptoral anchors and phylogeny of 14 species of *Ligophorus* from the Mediterranean Sea, Black Sea and Sea of Azov. This question has already been partly addressed by Khang et al. (2016) in 13 *Ligophorus* spp. from Malaysia, where strong correlation between anchor shape variation and phylogeny was found. However, Khang et al. (2016) tested the phylogenetic signal in anchor shape, but not in size, and did not evaluate the potential effect of evolutionary allometry on the phylogenetic signal (Monteiro, 1999; Klingenberg and Marugán-Lobón, 2013), whereas these aspects are deliberately addressed in the present effort. More importantly, their study was geographically constrained to the Malay Peninsula and involved two host species only. In fact, their *Ligophorus* spp. were distributed in two clades corresponding to host species and, therefore, it is difficult to determine whether, and to which extent, morphological differences between the two clades reflect phylogeny or adaptation to host species.

Our study model is more complex, involving six host species and several host-switches (Blasco-Costa et al. 2012; Sarabeev and Desdevises, 2014), allowing testing more elaborate hypotheses. For

instance, if adaptation to branchial morphology of the host species were a decisive driver of haptoral morphology, it would be expected that anchor form of the switched species differs substantially from that of their closest phylogenetic relatives and be similar to that of other species occurring on the same host species. Alternatively, if phylogeny were the major determinant, anchor morphology would remain relatively constant within the clade and will differ from that of more distant species co-occurring on the same host.

In this study we specifically use tools of geometric morphometrics that can be applied in the phylomorphospace and multivariate statistical tests with the aim of quantifying phylogenetic signal in shape and size in ventral and dorsal anchors in 14 species of *Ligophorus* in order to determine the relative weights of convergence and shared evolutionary history, driving anchor form within the genus. We illustrate how the search for phylogenetic signal in morphological traits combined with multivariate statistics can improve our understanding of evolutionary morphology in Monogenea and parasites in general

7.2. Materials and Methods

Ethics statement

The fishes needed for the study were obtained within day-to-day fishery operations and purchased dead from licensed commercial fishermen or local fish markets. The number of specimens of fish used (77) was kept to a reasonable minimum to guarantee the success of the research (see Table S1). Grey mullets are locally and globally abundant and are not subjected to special conservation regulations in Spain, Russia and Ukraine, and the species involved—*Mugil cephalus* L., 1758, flathead grey mullet, *Liza saliens* (Risso, 1810), leaping mullet, *Liza ramada* (Risso, 1827), thinlip grey mullet, *Liza aurata* (Risso, 1810), golden grey mullet, *Chelon labrosus* (Risso, 1827), thicklip grey mullet, and

Liza haematocheila (Temminck and Schlegel, 1845), so-iuy mullet—are listed by the IUCN as "Least Concern".

7.2.1. Sample composition

We based our morphological analysis on 286 individuals belonging to 14 of 16 valid species of *Ligophorus* (about 23% of all known species of the genus) recorded in the Mediterranean, Black Sea and Sea of Azov: *Ligophorus acuminatus* Euzet and Suriano, 1977; *Ligophorus cephali* Rubtsova, Balbuena, Sarabeev, Blasco-Costa and Euzet, 2006; *Ligophorus chabaudi* Euzet and Suriano, 1977; *Ligophorus confusus* Euzet and Suriano, 1977; *Ligophorus heteronchus* Euzet and Suriano, 1977; *Ligophorus imitans* Euzet and Suriano, 1977; *Ligophorus macrocolpos* Euzet and Suriano, 1977; *Ligophorus mediterraneus* Sarabeev, Balbuena and Euzet 2005; *Ligophorus minimus* Euzet and Suriano, 1977; *Ligophorus szidati* Euzet and Suriano, 1977; *Ligophorus vanbenedenii* Euzet and Suriano, 1977; *Ligophorus llewellyni* Dmitrieva et al. 2007; *Ligophorus pilengas* Sarabeev and Balbuena, 2004 and *Ligophorus angustus* Euzet and Suriano, 1977. The sample size for each species was 20 individuals for ventral and 20 individuals for dorsal anchors (not necessarily matching specimens of the previous group), except in *L. angustus*, where only 4 individuals for ventral and none for dorsal anchors could be studied, and so dorsal anchor was left out of the analysis for this species. In all, 524 anchors were studied of which, in 238 instances, represented ventral and dorsal anchors of the same worm individual.

The present study covers all six grey mullets species reported as host of *Ligophorus* spp. in the Mediterranean, Black Sea and Sea of Azov, including the so-iuy mullet *Liza haematocheila*, which was introduced in the Black Sea and Sea of Azov from the Pacific in the early 1980s (Sarabeev et al. 2015).

The parasite specimens were collected in the frame of previous studies of our group (Blasco-Costa et al. 2012; Sarabeev et al. 2013; Sarabeev and Desdevises, 2014; Llopis-Belenguer et al. 2015; Rodríguez-González et al. 2015b; Rodríguez-González et al. 2016) in two marine areas of the Spanish Mediterranean Coast (the Ebro Delta, and Santa Pola Bay), a coastal Mediterranean lagoon (L'Albufera), and the Sea of Azov (Kerch Strait). In addition, part of the specimens of *L. llewellyni* and *L. pilengas* were collected in the Sea of Japan (Artemovka Delta), i.e., in the host's native area. (Geographical details of all localities are given in Table 7S1). Gills were examined for parasites as per Rodríguez-González et al. (2015b).

7.2.2. Geometric morphometrics

7.2.2.1. Morphological data acquisition and landmarks superimposition

Only the anchors were considered for geometric morphometrics techniques because they are not subjected to large variation due to contraction or flattening on fixation (Lim and Gibson, 2009). The bars were not studied because they are more difficult to observe flat and more prone to distortion during fixation and mounting. We used photographs and drawings only for ventral and dorsal anchors of partly digested individuals following Rodríguez-González et al. (2015b). Any anchor showing apparent deformation, tear or rupture (about 2-3% of the initial sample) was excluded from the study.

Although Khang et al. (2016) provided an ad hoc quality-control method for haptoral anchors, we preferred the use of the tool provided in MorphoJ v.1.06d Klingenberg (2011) to detect morphological outliers. Mostly, because their method measures discrepancies between right and left forms, which implies that specimens showing fluctuating asymmetry can be confounded with poor quality specimens (Khang et al. 2016). Although the authors assumed that it was not common in their sample, fluctuating asymmetry has indeed been shown to occur in *L. cephali* (Llopis-Belenguer et al. 2015). Accordingly, we compared the cumulative distribution of the distances of

individual specimens from the average shape of the entire sample with the curve expected for a multivariate normal distribution fitted to the data. The stretched-to-the-right empirical distributions obtained (Figure 7S1) indicated that none of the specimens chosen, deviated manifestly from the others.

Anchor shape was characterized using landmark-based geometric morphometrics (Klingenberg, 2010). We digitized 8 landmarks in 2D covering the anchor surface selected and recorded in each anchor using tpsDig version 2.17 (Rohlf, 2015) representing homologous points (see Figure 4.1 in Rodríguez-González et al. (2015b). Generalized Procrustes analysis in MorphoJ was employed to obtain a matrix of shape coordinates from which all information related to position, scale and orientation were removed (Dryden and Mardia, 1998). Centroid size, the summed squared distances of each landmark from the centroid of the form was used as a measure of size (Zelditch et al. 2012). The covariance matrices generated of landmark data of ventral and dorsal anchors, were subjected to a Principal Component Analysis (PCA). To visualize the variation in shape, we used the first two principal components (PC1 and PC2).

7.2.3. Quantifying the influence of size on anchor shape

The effects of size on interspecific variation in anchors shape of *Ligophorus* spp. (i.e. interspecific allometry) were tested separately for ventral and dorsal anchors by multivariate regression analyses (Monteiro, 1999). We regressed the Procrustes shape coordinates of ventral and dorsal anchors on their log-transformed centroid size (logCS) by means of a multivariate regression through the origin (Klingenberg and Marugán-Lobón, 2013; Klingenberg et al. 2012). Then, we mapped the residuals from this regression onto the phylogenetic tree of the parasites. A large difference between the original datasets and the residuals would indicate that evolutionary allometry is an important factor in anchors evolution in *Ligophorus*.

The effect of size on shape was also assessed with phylogenetic independent contrast (PIC) correction (Felsenstein, 1985) in order to avoid incorrect interpretations due to a violation of the assumption of independent sampling (Harvey and Pagel, 1991). However, no evidence for allometry in any of the PIC-corrected analyses was found significant ($P > 0.3$ in both cases) and, therefore, the effect of evolutionary allometry was not further considered.

7.2.4. Assessing phylogenetic signal in anchor shape and size

Phylogenetic signal was assessed by mapping a topology of the phylogenetic tree of our 14 species of *Ligophorus* based on a previous published concatenated 28S rDNA and ITS1 phylogeny (Rodríguez-González et al. 2016) onto the first two principal component scores of shape and size-corrected shape, and onto logCS representing anchor size. This required an ancestral state reconstruction of the morphometric data for each internal node on the tree using squared change-parsimony assuming a Brownian-motion model of evolution (Klingenberg and Marugán-Lobón, 2013).

Phylogenetic signal was tested with MorphoJ (Klingenberg, 2011), where the sum of squared changes of shape along the branches of the tree is minimized over the entire phylogeny. The significance of phylogenetic signal was established by a permutation test in which the topology was held constant and the principal component scores for each taxon were randomly permuted 10,000 times across the tree (Maddison, 1991; Klingenberg and Gidaszewski, 2010).

The previous analyses provided values of tree length that are inversely related to the strength of the correlation between shape or size and phylogeny (Klingenberg and Gidaszewski, 2010). In addition, due to the current controversy on which method is more appropriate to evaluate phylogenetic signal (Adams, 2014), we also used K_{mult}, which is a generalization of Blomberg's K (Adams, 2014; Adams and Otárola-Castillo, 2013). The main advantage of this approach is that, in addition to informing whether there is a small or large amount of signal present in data, they

provide a reference value for departure from the Brownian-motion model of evolution (Diniz-Filho, 2012). K_{mult}= 0 indicates no phylogenetic signal, K_{mult}= 1 corresponds to phylogenetic signal in the data and that the trait distribution perfectly conforms to the Brownian's model of trait evolution, values of K_{mult} < 1 correspond to phenotypic variation that is larger than expected between taxa of the same lineage, and K_{mult} > 1 indicates stronger similarities among closely related species than expected under the Brownian's model. The significance of K_{mult} was evaluated based on comparison of the observed value with those obtained in 999 randomizations (Liu et al. 2015). The calculation were performed with function *physignal* in the geomorph package v.3.0.1. (Adams, 2014) in R version 3.2.3 (Development Core Team, 2014).

7.3. Results

7.3.1. Phylogenetic signal in anchor shape and anchor size

The PCA based on the covariance matrix of landmark data of both ventral and dorsal anchors showed that a large proportion of the variation is contained in relatively few dimensions, with the first two PCs accounting for over a half of the total variance in the sample (Table 7.1). The first two axes described 69.9% and 52.9% of the total shape variation (uncorrected for size) and 66.4% and 51.8% of the total shape variation (size-corrected) in ventral and dorsal anchors, respectively (eigenvalues and variance explained by each principal component are given in Tables 7S2 and 7S3). The anchor shapes in our sample were distributed in all shape tangent space for both ventral and dorsal anchors, which are surrounded by distant species from the average shape.

Table 7.1 PCA of variation among the shapes of species mean for ventral and dorsal anchors of *Ligophorus* spp. for original and size-corrected shape.

	Size-uncorrected		Size-corrected	
Anchor	Eigenvalue	Total variance (%)	Eigenvalue	Total variance (%)
Ventral				
PC1	$1.23 \cdot 10^{-2}$	56.6	$1.07 \cdot 10^{-2}$	53.7
PC2	$2.90 \cdot 10^{-3}$	13.3	$2.52 \cdot 10^{-3}$	12.7
Dorsal				
PC1	$5.08 \cdot 10^{-3}$	36.9	$4.85 \cdot 10^{-3}$	36.4
PC2	$2.20 \cdot 10^{-3}$	15.9	$2.04 \cdot 10^{-3}$	15.3

The molecular phylogeny of *Ligophorus* spp. projected onto the morphospace defined by the first two PCs of the ventral and dorsal anchor shape is shown in Figure 7.1. This resulted, respectively, in tree lengths of 0.045 and 0.027, measured in units of squared Procrustes distance along all branches. The deformation grids of each species showing departure from the average anchor shape are also shown.

The projection of the phylogenetic trees onto the morphospaces of ventral and dorsal anchors (Figure 7.1) showed crossing of branches and some evidence of relatively long branches between related species for ventral and dorsal anchors of species of *Ligophorus*. However, the permutation tests of PC scores revealed significant phylogenetic structure for shape in both ventral and dorsal anchors ($P < 0.0001$ in both cases). Likewise, the K_{mult} values were significantly greater than zero (ventral anchors: $K_{mult} = 0.99$, $P = 0.001$; dorsal anchors: $K_{mult} = 0.35$, $P = 0.037$). In fact, in both ventral and dorsal anchors clades occupied specific regions of shape space, which is indicative of phylogenetic structure in the data (Klingenberg and Marugán-Lobón, 2013) (Figure 7.1). In ventral anchors, interspecific variation was caused by the different position of anchors of different clades (Figure 7.1A). The clade formed by *L. confusus*, *L. szidati* and *L. angustus* was characterized

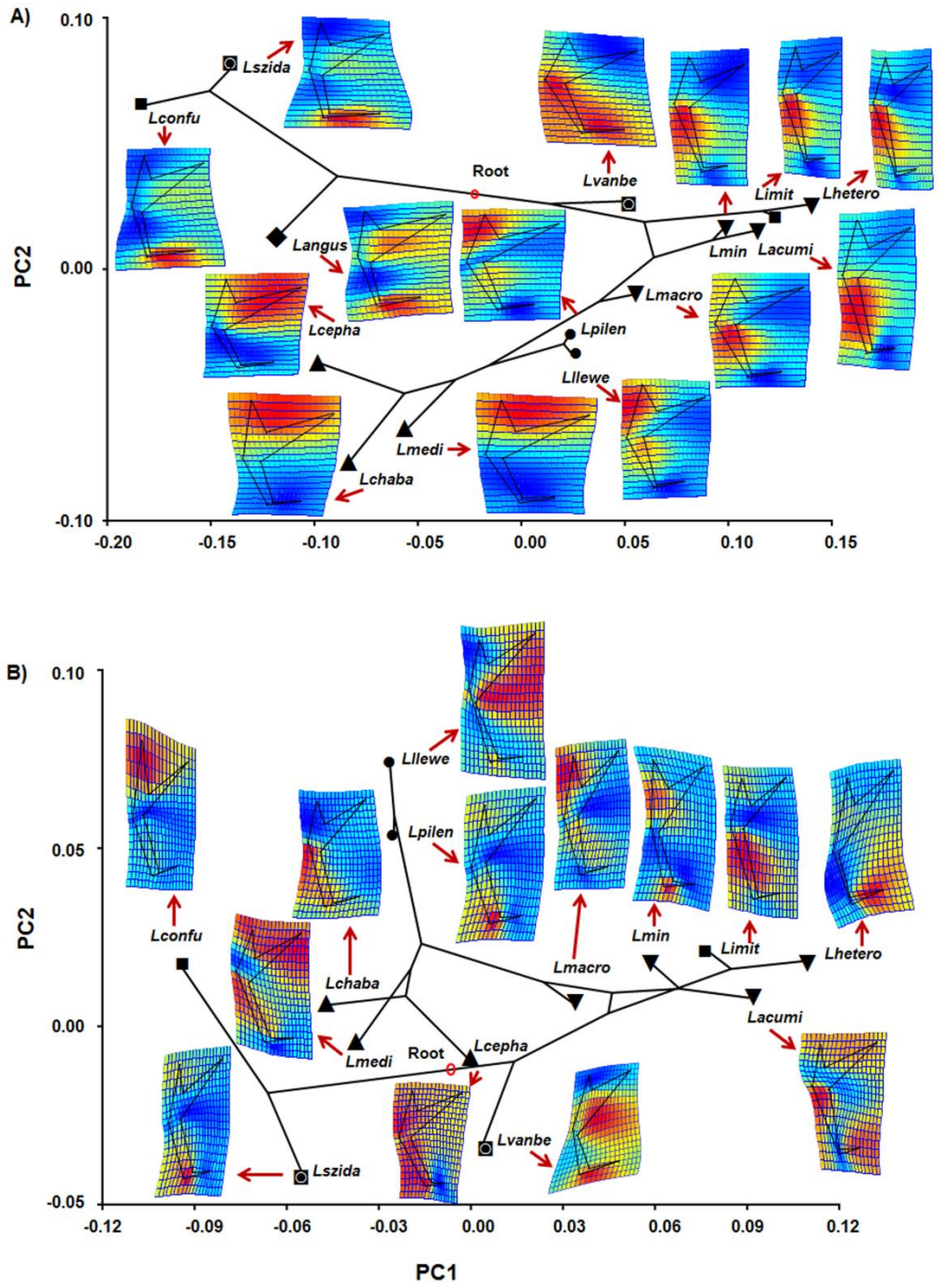

Figure 7.1. Projection of phylogeny of 14 species of *Ligophorus* spp. onto the morphospaces of ventral A) and dorsal anchors B). Estimated changes in anchor shapes are shown as Thin-plate-spline deformation grids with color-scaled coded Jacobian expansion factors (red for factors > 1, indicating expansion; strong blue for factors between 0 and 1, indicating contraction) were used. The ventral anchors of all species included in the analysis are labeled: *Lconfu*: *Ligophorus confusus*, *Lszida*: *Ligophorus szidati*, *Langus*: *Ligophorus angustus*, *Lvanbe*: *Ligophorus vanbenedenii*, *Limit*: *Ligophorus imitans*, *Lhetero*: *Ligophorus heteronchus*, *Lacumi*: *Ligophorus acuminatus*, *Lmin*: *Ligophorus minimus*, *Lmacro*: *Ligophorus macrocolpos*, *Lpilen*: *Ligophorus pilengas*, *Lllewe*: *Ligophorus llewellyni*, *Lmedi*: *Ligophorus mediterraneus*, *Lchaba*: *Ligophorus chabaudi*, *Lcepha*: *Ligophorus cephali* and their host as: *Liza saliens* (▼), *Chelon labrosus* (◆), *Liza ramada* (■), *Liza haematocheila* (●), *Liza aurata* (◙) and *Mugil cephalus* (▲).

by a long point, short shaft and long inner root, the three species occur each on different hosts (*Liza ramada*, *Liza aurata* and *Chelon labrosus*, respectively). A second clade formed by *L. cephali*, *L. chabaudi* and *L. mediterraneus* from *M. cephalus*, and by *L. pilengas* and *L. llewellyni* from *Lz. haematocheila* was characterized by large outer roots and short points. Within this clade the anchors of species on *M. cephalus* could be distinguished from those occurring on *Lz. haematocheila* by the larger outer roots. Two other clades comprising *L. imitans* and *L. heteronchus*, and *L. acuminatus* and *L. minimus*, together with *L. macrocolpos* exhibited elongated ventral anchors with short points, relatively short inner and outer roots and long shafts. These species are found on *Liza saliens*, except *L. imitans*, that occurs on *Lz. ramada*. Finally, the shape of anchors of *L. vanbenedenii* occurring on *Lz. aurata* is intermediate between that of the last five species and that of the *L. confusus*–*L. angustus* clade, which is consistent with the phylogenetic position of this species (Figure 7.1A). In contrast to ventral anchors and although the spatial arrangement of clades in the morphospace was very similar, shape variation in dorsal anchors was more unpredictable as the deformation grids showed quite different patterns at the species level (Figure 7.1B). As a result, specific shapes could not be clearly associated with particular clades.

The phylogeny projected onto the first two dimensions of the allometry-free (size-corrected) PCA morphospace of anchor shape yielded tree lengths of 0.04 and 0.02 for ventral and dorsal anchors, respectively (Figure 7.2). The highly significant multivariate regression of Procrustes coordinates on logCS ($P < 0.001$) provided evidence for allometric relationships between shape and size in both types of anchors. This relationship accounted for 9.2% and 4.9 % of the total shape variation of ventral and dorsal anchors respectively. Again phylogenetic signal was highly significant ($P < 0.0001$ and $P = 0.0015$ respectively). According to the phylogenetic signal with K_{mult} (size-corrected), the results were significant (ventral: $K_{mult} = 0.76$, $P = 0.001$; dorsal: $K_{mult} = 0.77$,

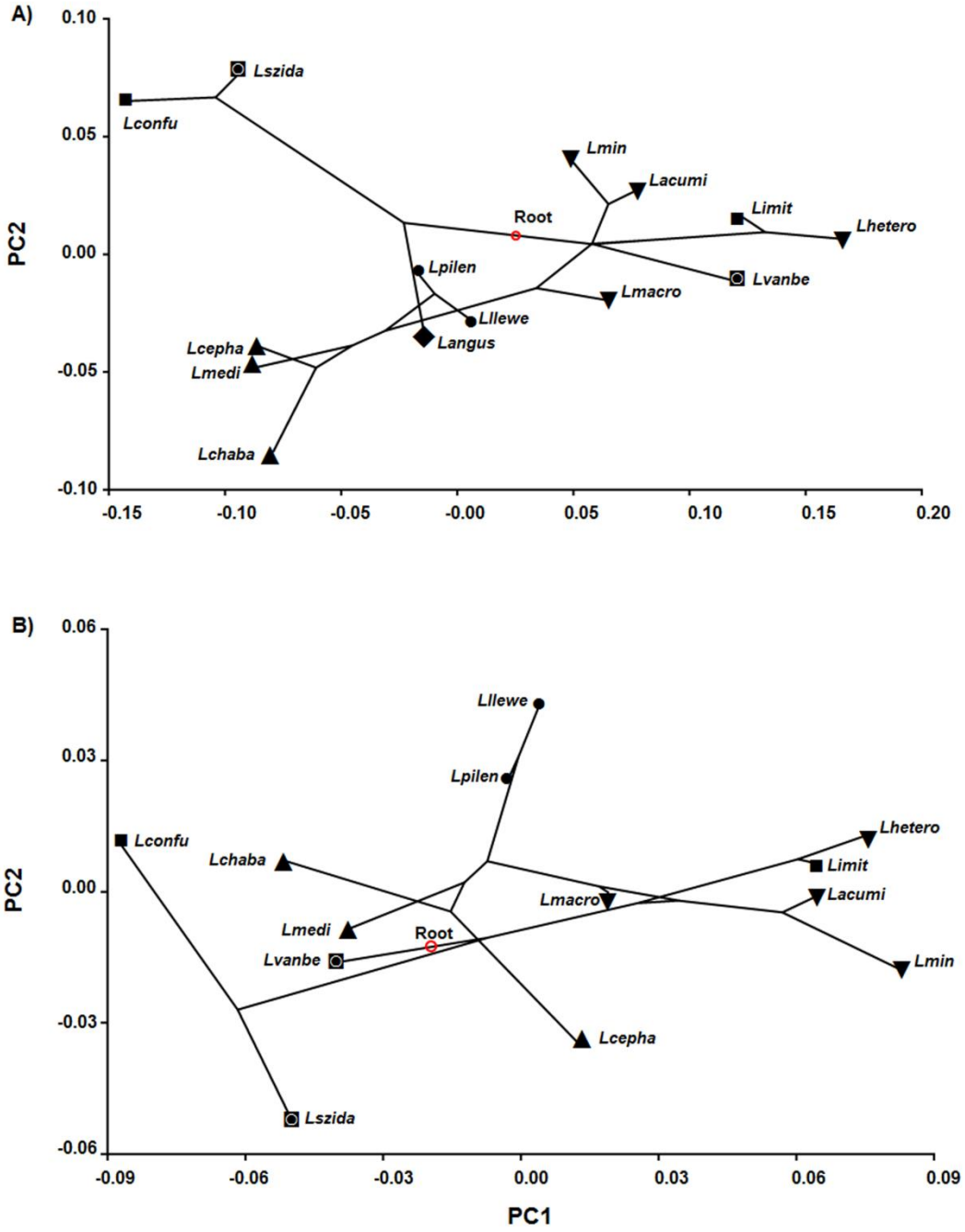

Figure 7.2. Projection of phylogeny of 14 species of *Ligophorus* spp. onto the morphospaces corrected for size of ventral A) and dorsal anchors B). Species abbreviations and host symbols as in Figure 7.1.

P= 0.002). The scatterplot of ventral anchors (Figure 7.2A) showed larger branches of *L. angustus*, *macrocolpos*, *L. vanbenedenii*, and *L. heteronchus* than in the PCA uncorrected for size (Figure 7.1A). Likewise, for the dorsal anchors (Figure 7.2B), the branches of *L. szidati*, *L. confusus*, *L. cephali*, *L. chabaudi*, *L. minimus*, *L. llewellyni* and *L. heteronchus* were larger than the original PCA (Figure 7.1B). However, in both cases the position of species in the shape space was similar to the arrangement shown in Figure 7.1. Therefore allometry had a moderate effect on the overall variation of anchors shape.

The molecular phylogeny projected onto the gradient in size (logCS) of ventral and dorsal anchors is shown in Figure 7.3, where the cumulative branch length from the root of the tree is displayed vertically. This mapping resulted in tree lengths of 0.048 and 0.077, for ventral and dorsal anchors respectively, measured in units of logCS distance along all branches. In ventral anchors, *L. angustus* showed the larger branches and were separated from all other species, indicating a smaller anchor size than in the other species. Phylogenetic signal tested by random permutation of logCS was statistically significant ($P < 0.001$) in ventral anchors (Figure 7.3A), but not in dorsal ones (Figure 7.3B) (P= 0.271), whereas Adams (2014) K_{mult} indicated a significant phylogenetic signal in both anchors (ventral: K_{mult}= 1.34, P= 0.001; dorsal: K_{mult}= 0.99, P= 0.003).

7.4. Discussion

This paper delivers a novel framework to study the evolution of attachment organs in monogeneans and paves the way for further studies addressing the relationships among morphology, phylogeny and other traits, such as host specificity or niche occupancy. Patterns of morphological change in haptoral anchors were interpreted to reconstruct the dynamics of the evolutionary processes and were visualized as paths from ancestors to descendants through the morphospace.

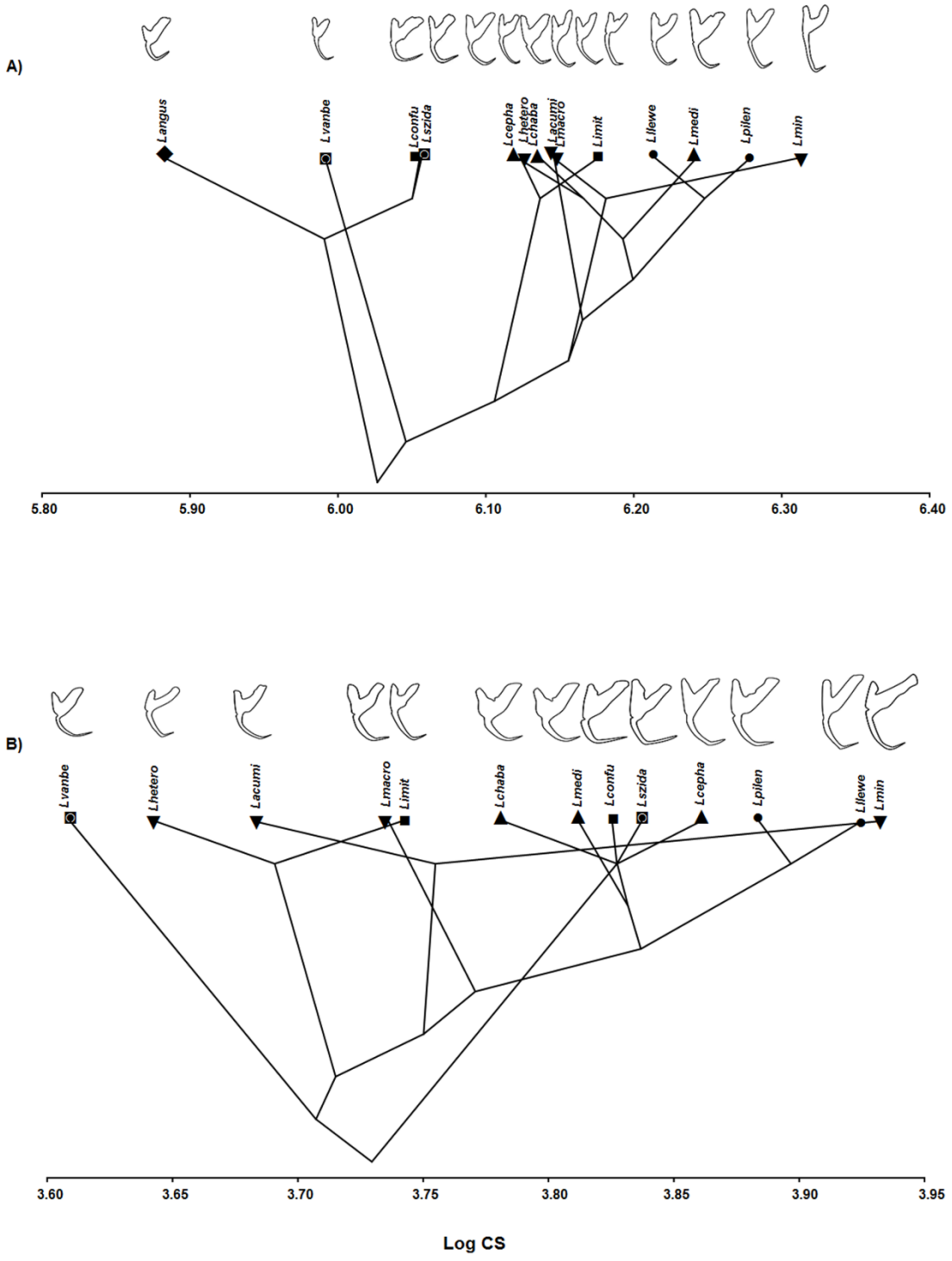

Figure 7.3. Projection of phylogenetic tree of 14 *Ligophorus* spp. onto *log* Centroid Size (LogCS) of ventral A) and dorsal B) anchors. Species abbreviations and host symbols as in Figure 7.1. The anchors displayed are scaled as per the LogCS scale to convey the gradient in size.

Given the variety of anchors shapes in *Ligophorus* (Sarabeev et al. 2013); it is not surprising that they cover a substantial range of shapes in the tangent space (Figures 7.1 and 7.2). The tests performed provided strong evidence for phylogeny playing a major role in determining the shape and, to a lesser degree, the size of the haptoral anchors, which fully agrees with previous work showing a consistent relationship between morphology and phylogeny in *Ligophorus* (Blasco-Costa et al. 2012; Sarabeev and Desdevises, 2014; Khang et al. 2016).

Many monogeneans, including the members of *Ligophorus*, are known to be highly host-specific (Sarabeev et al. 2013), which implies a close interaction with their host. Given that the host can have an influence on genetic and morphological differentiation of monogeneans (Desdevises et al. 2002), it has been often hypothesised that haptor morphology reflects adaptations to attachment to the host (Šimková et al. 2002). This hypothesis can be assessed in this geometric morphometrics framework by comparing the position in the phylomorphospace of distantly related species co-occurring on a given host species. *L. confusus* and *L. imitans* parasitizing *Lz. ramada* represent different clades and their anchors fell far apart in the shape and size morphospaces. Similarly, *L. szidati* and *L. vanbenedenii* co-occurring on *Lz. aurata*, and placed in different clades, differed markedly in shape of the dorsal anchor and size of the dorsal anchor (Figures 7.1 and 7.2). Therefore, we found no clear evidence for host-driven homoplasy in the *Ligophorus* spp. studied. However, only these two instances could be analysed and, as discussed below, specific positions in the gills by each species should also be considered.

Ligophorus and Mugilidae define an interesting scenario of host parasite associations. Each species of *Ligophorus* predominantly occurs on a single host species and that often co-occurs with one or more congeneric species (Blasco-Costa et al. 2012). In several instances, members of clades that occur on the same host species showed similar anchor forms (*L. cephali* – *L. mediterraneus* on *M. cephalus*, *L. llewellyni* and *L. pilengas* on *Lz. haematocheila*) or similar shapes (*L. acuminatus* and *L. minimus*

on *Lz. saliens*). These clades probably resulted from several synxenic speciation events (Huyse et al. 2005).

In addition, sister species occurring on different hosts showed similarities in shape, sometimes also in size, of anchors (compare, for instance, anchor forms of *L. imitans* and *L. szidati* with those of their respective sister species *L. heteronchus* and *L. confusus* (Figures 7.1 and 7.2). The phylogenetic position of *L. imitans*, showing affinities with species found on *Lz. saliens*, suggests that its occurrence on *Lz. ramada* represents a host-switch. The most ancestral clade formed by *L. angustus*, *L. confusus* and *L. szidati* is also result of host-switch evolutionary events, as each monogenean species of the clade occurs on different mullet hosts. So adaptation to a new host species did not impose dramatic changes in haptoral anchor morphology and the morphological similarities observed point to the occurrence of phylogenetic constrains on anchor form, as proposed for other monogeneans, such as *Lamellodiscus* spp. (Desdevises et al. 2002) and *Cichlidogyrus* spp. (Mendlová and Šimková, 2014).

Our geometric morphometrics approach also allows identifying paths of morphological evolution. For example, within the *L. heteronchus* – *L. cephalus* clade (corresponding to clade II of Blasco-Costa et al. (2012)), the basal species (*L. heteronchus* to *L. macrocolpos*, predominantly associated to *L. saliens*, possess narrow anchors with long shafts. This shape would therefore represent the ancestral state relative to the morphologically derived anchors of the *L. llewellyni* – *L. cephalus* clade, which includes forms on *Lz. haematocheila* and *M. cephalus* characterized by larger roots. Roots provide the bases for muscle attachment, so that the force is exerted through muscles and transmitted to the point controlling the anchor grip strength on the gills (Rodríguez-González et al. 2016). Given that *Lz. haematocheila* and *M. cephalus* represent the largest host species in the present study (Froese and Pauly, 2016), one can venture the hypothesis that larger roots were evolved for greater grip in order to withstand stronger water currents (Sarabeev and Desdevises, 2014). In any

case, the similarities in anchor morphology of the species occurring on *M. cephalus* with those occurring on the Pacific *Lz. haematocheila* support the idea that the occurrence of *Ligophorus* in *Mugil* can be explained by a host-switch from the *Liza–Chelon* clade that occurred outside the Mediterranean basin (Blasco-Costa et al. 2012).

The evidence for phylogenetic signal was more compelling for the ventral anchors, than for the dorsal ones. This is perhaps not surprising given that dorsal and ventral anchors in *Ligophorus* form two relatively independent evolutionary modules (Rodríguez-González et al. 2016). Empirical evidence from *L. cephali* indicates a tighter control of the shape and size in ventral anchors perhaps because they seem to be responsible for firmer attachment (Llopis-Belenguer et al. 2015; Rodríguez-González et al. 2015b). Thus the differences observed could be explained in terms of different functional roles in attachment to the gills (Rodríguez-González et al. 2016). In the present study, the K_{mult} corresponding to the shape of dorsal anchors was clearly < 1, which indicates that phenotypic variation is larger than expected between taxa of the same lineage (Adams, 2014). It has been suggested that a certain degree of homoplasy could account for low K_{mult} values of anchor shape in monogeneans (Khang et al. 2016). Although the deformation grids do not provide clear evidence for this (Figure 7.1B), there might still be some hidden homoplasy at the level of within-host microhabitats. Microhabitat was not considered in the present effort because information concerning *Ligophorus* spp. is very scarce (Sanfilippo, 1978; Euzet and Sanfilippo, 1983; Pronkina et al. 2010). Previous work has shown that *L. szidati* and *L. vanbenedenii* on *Lz. aurata*, and *L. parvicirrus* on *Lz. ramada* differ in their location in the gills (Sanfilippo, 1978; Euzet and Sanfilippo, 1983; Pronkina et al. 2010) and, as representatives of different clades, possess distinct morphologies of their attachment organs as discussed above. In addition, Rodríguez-González et al. (2015b) showed that random effects such as gill section-host individual are important determinants of shape variation in ventral anchors in *L. cephali*. So a combination of host species, individual host and microhabitat

might contribute to explain the high diversity of dorsal anchor shapes observed (Figure 7.1B). In any case, if microhabitat information becomes available, it can be readily incorporated into the analyses and future studies of monogeneans can greatly benefit from this approach.

In this study, we have demonstrated that variation of shape and size of the ventral and dorsal anchors in 14 *Ligophorus* spp. is largely determined by common descent and shared evolutionary history, although homoplasy dictated by adaptations to the individual host or to specific gill microhabitats could not be ruled out completely. These two processes may act differently in other monogeneans, but similar analyses of variation in haptoral form as those presented herein can decisively contribute to our understanding of the evolution of attachment organs in monogeneans (Šimková et al. 2006; Vignon and Sasal, 2010; Mendlová and Šimková, 2014; Khang et al. 2016; Kmentová et al. 2016) and other parasites in general. In particular, the adoption of the present approach can help bridge the gap between micro and macroevolutionary processes. Haptoral morphology determines, within one individual host, the specific microhabitats on the gills that, in turn, can influence the specialization and the reproductive isolation among conspecifics through niche segregation (Morand et al. 2002; Šimková et al. 2002; Jarkovský et al. 2004). We therefore expect that the present work stimulates further investigations in this area.

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Author Contributions

AR-G, JAB conceived and designed the experiments. AR-G, JAB performed the experiments. AR-G analyzed the data. AR-G, VS, JAB contributed reagents/materials/analysis tools. AR-G, JAB wrote the paper.

Competing interests

The authors declare that no competing interests exist.

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Supplementary material

Table 7S1. Species of *Ligophorus* used in this study collected from five localities: Ebro Delta (40°30′–40°50′N, 0°30′–1°10′E); Santa Pola Bay (38°00′–38°20′N, 0°10′–0°40′W); L'Albufera (39°20′0″N–0°21′0″W); Kerch Strait, Sea of Azov (45°16′20.8″N–36°31′40.6″E); and Artemovka Delta, Sea of Japan (43°18′30.3″N–132°17′4.8″E).

Species of *Ligophorus*	*NV*	*ND*	Host species	Number of fishes	Ebro Delta	Santa Pola	L'Albufera	Kerch Strait	Artemovka Delta
Ligophorus acuminatus Euzet and Suriano, 1977	20	20	*Liza saliens*	7	X				
Ligophorus cephali Rubtsova, Balbuena, Sarabeev, Blasco-Costa and Euzet, 2006	20	20	*Mugil cephalus*	20			X		
Ligophorus chabaudi Euzet and Suriano, 1977	20	20	*Mugil cephalus*	4	X	X			
Ligophorus confusus Euzet and Suriano, 1977	20	20	*Liza ramada*	5		X			
Ligophorus heteronchus Euzet and Suriano, 1977	20	20	*Liza saliens*	3	X				
Ligophorus imitans Euzet and Suriano, 1977	20	20	*Liza ramada*	3	X	X			
Ligophorus macrocolpos Euzet and Suriano, 1977	20	20	*Liza saliens*	4	X	X			
Ligophorus mediterraneus Sarabeev, Balbuena and Euzet, 2005	20	20	*Mugil cephalus*	3	X	X			
Ligophorus minimus Euzet and Suriano, 1977	20	20	*Liza saliens*	7	X		X		
Ligophorus szidati, Euzet and Suriano, 1977	20	20	*Liza aurata*	4		X			
Ligophorus vanbenedenii Euzet and Suriano, 1977	20	20	*Liza aurata*	5		X			
Ligophorus llewellyni Dmitrieva, Gerasev and Pron'kina, 2007	20	20	*Liza haematocheila*	4				X	X
Ligophorus pilengas Sarabeev and Balbuena, 2004	20	20	*Liza haematocheila*	5				X	X
Ligophorus angustus Euzet and Suriano, 1977	4	0	*Chelon labrosus*	3	X				

NV, number of ventral anchors; *ND*, number of dorsal anchors.

Table 7S2. Eigenvalues and associated percent of variance accumulated for each principal component (PC1-12) of shape Principal Component Analyses of ventral anchors corrected and uncorrected for size.

Ventral anchors	Size-uncorrected			Size-corrected		
	Eigenvalues	% Variance	Cumulative %	Eigenvalues	% Variance	Cumulative %
PC 1	0.01231297	56.60	56.60	0.0106649	53.71	53.71
PC 2	0.0028931	13.30	69.90	0.0025165	12.67	66.38
PC 3	0.0018328	8.43	78.33	0.0018409	9.27	75.65
PC 4	0.0011447	5.26	83.59	0.0011521	5.80	81.45
PC 5	0.0010554	4.85	88.44	0.0010759	5.42	86.87
PC 6	0.0007714	3.55	91.99	0.0007546	3.80	90.67
PC 7	0.0005305	2.44	94.43	0.0006061	3.05	93.72
PC 8	0.0004351	2.00	96.43	0.0004564	2.30	96.02
PC 9	0.0002761	1.27	97.69	0.000281	1.42	97.43
PC 10	0.0002548	1.17	98.87	0.0002624	1.32	98.75
PC 11	0.0002039	0.94	99.80	0.0002051	1.03	99.79
PC 12	4.296E-05	0.20	100.00	4.243E-05	0.21	100.00

Table 7S3. Eigenvalues and associated percent of variance accumulated for each principal component (PC1-12) of shape Principal Component Analyses of dorsal anchors corrected and uncorrected for size.

Dorsal anchors	Size-uncorrected			Size-corrected		
	Eigenvalues	% Variance	Cumulative %	Eigenvalues	% Variance	Cumulative %
PC 1	0.0050842	36.93	36.93	0.0048569	36.48	36.48
PC 2	0.0021978	15.96	52.89	0.0020427	15.34	51.82
PC 3	0.0017967	13.05	65.94	0.0017909	13.45	65.28
PC 4	0.0015204	11.04	76.99	0.0015204	11.42	76.70
PC 5	0.0011129	8.08	85.07	0.0010701	8.04	84.73
PC 6	0.000563	4.09	89.16	0.0005448	4.09	88.83
PC 7	0.0004479	3.25	92.41	0.0004466	3.36	92.18
PC 8	0.0003742	2.72	95.13	0.0003731	2.80	94.98
PC 9	0.000272	1.98	97.11	0.0002719	2.04	97.02
PC 10	0.0001805	1.31	98.42	0.0001803	1.35	98.38
PC 11	0.0001676	1.22	99.63	0.0001664	1.25	99.63
PC 12	5.037E-05	0.37	100.00	4.956E-05	0.37	100.00

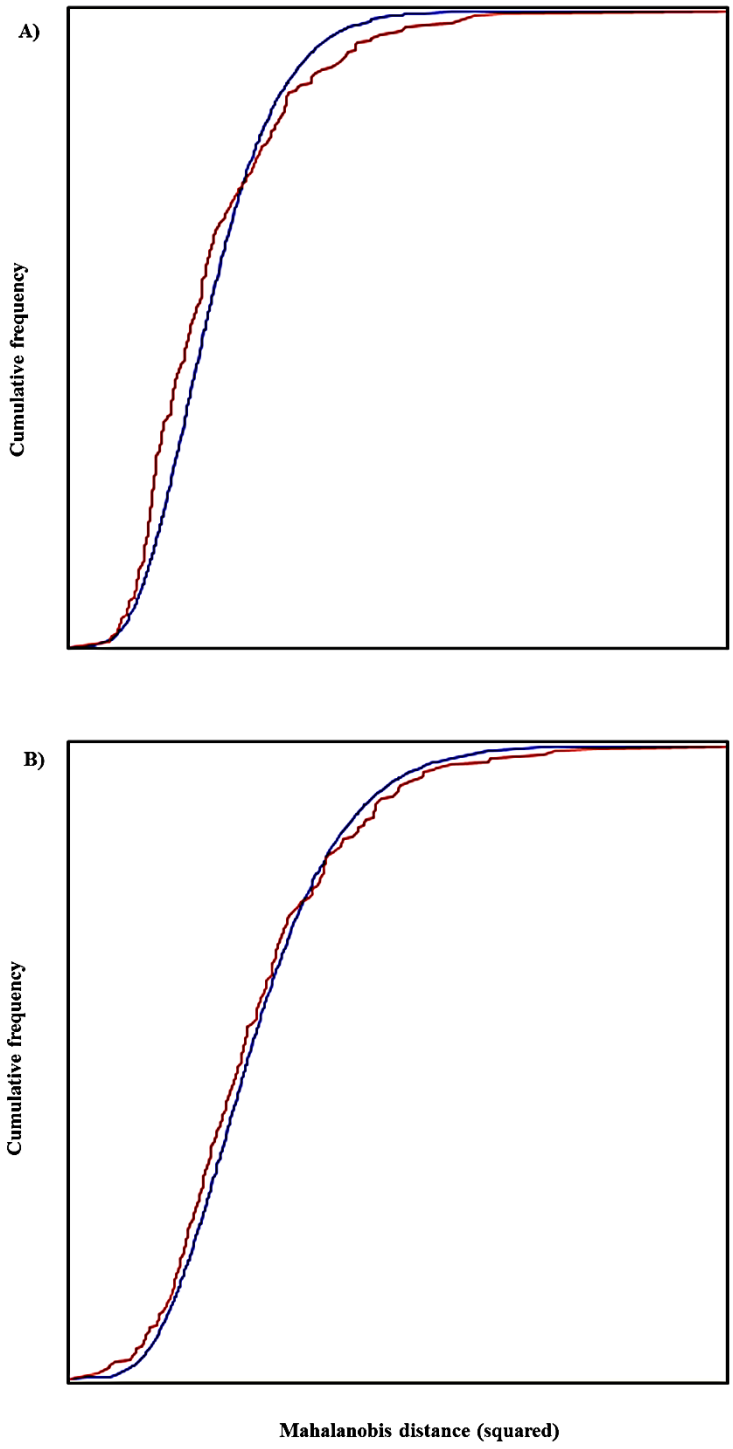

Figure 7S1 Diagrams provided by MorphoJ of the cumulative distribution of the distances of individual anchors from average anchor shape of the entire sample. The cumulative distribution (red curve) is compared with that expected for a multivariate normal distribution fitted to the data (blue curve). A) Ventral anchors and B) dorsal anchors.

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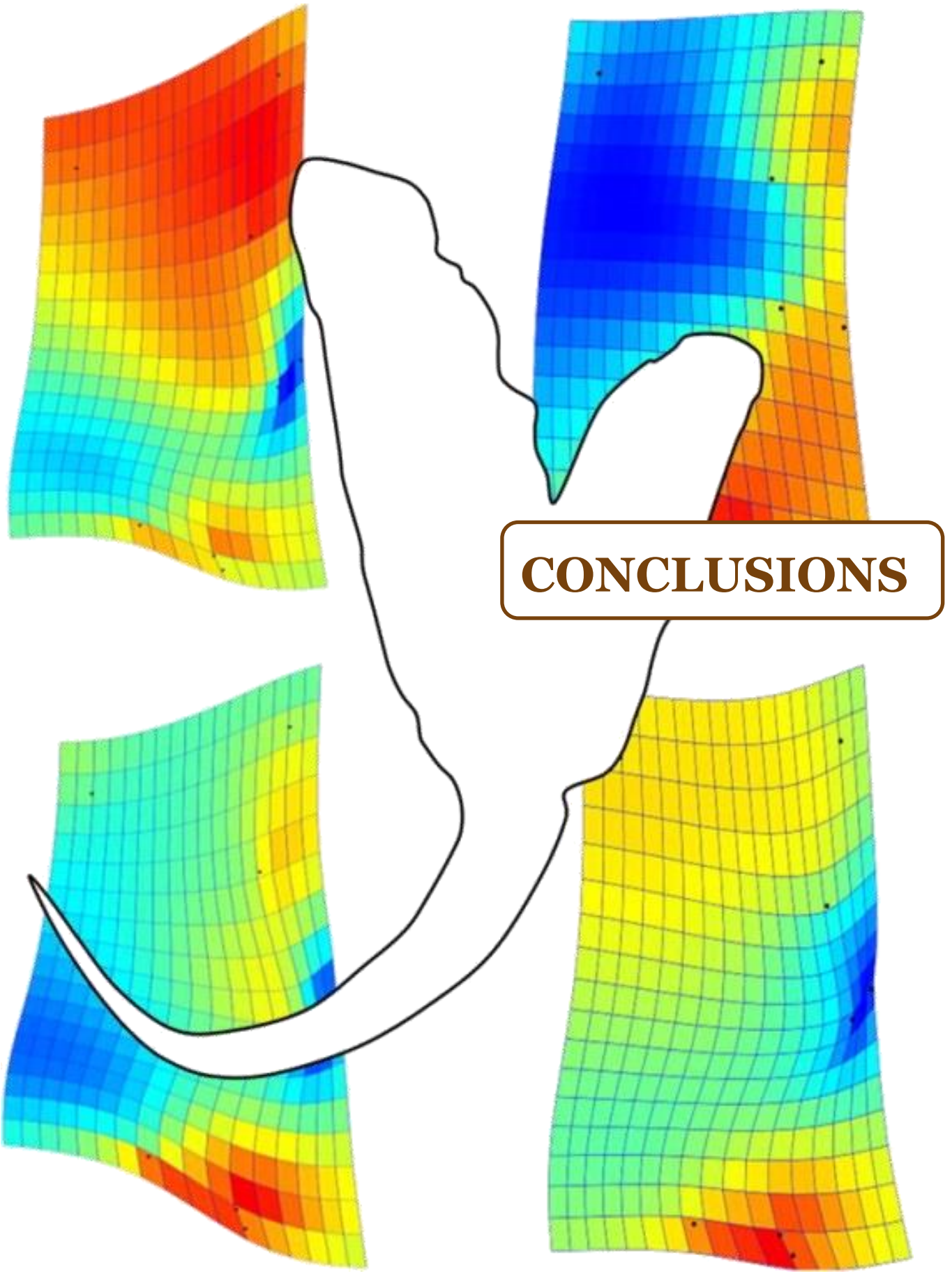


CONCLUSIONS

The present study used geometric morphometrics to provide new insights into the functional morphology of the anchors in *Ligophorus* spp. and the evolutionary processes driving morphological variation in these attachment organs. As a result of this study, the following main conclusions can be drawn:

1. Patterns of shape variation were similar in ventral and dorsal anchors. The localized variation was much higher in the dorsal anchors, which matched with high residual variation in the shape models. Random effects (section gill × single host) were an important determinant in the ventral anchors, but not so much in the dorsal anchors. The size models in the anchors were different. The dorsal anchor/bar complex seems more mobile than ventral one in *Ligophorus*, and these differences may reflect different functional roles in attachment to the gills.
2. The gill arch was an important determinant of anchor shape and size of the dorsal anchors. The shape variability of the form can be associated with the hydrodynamic processes that are associated with the spatial position of each gill and this can determine the leverage applied for attachment.
3. Phenotypic plasticity in anchor morphology of *Ligophorus* spp. could indicate the ability to colonize new hosts. Moreover, we found no evidence of correlation between dorsal or ventral anchors size and host size.
4. *Ligophorus yucatanensis* represents a new species from the gills of the flathead mullet *Mugil cephalus* from the Yucatan Peninsula. *L. yucatanensis* resembles more closely species from the Mediterranean Sea and off the coast of the northwestern Pacific than species recorded in South and North America and, according to zoographic records of *Ligophorus* spp. it is assigned to entity 4 (Western North Atlantic) of the *M. cephalus* species complex.

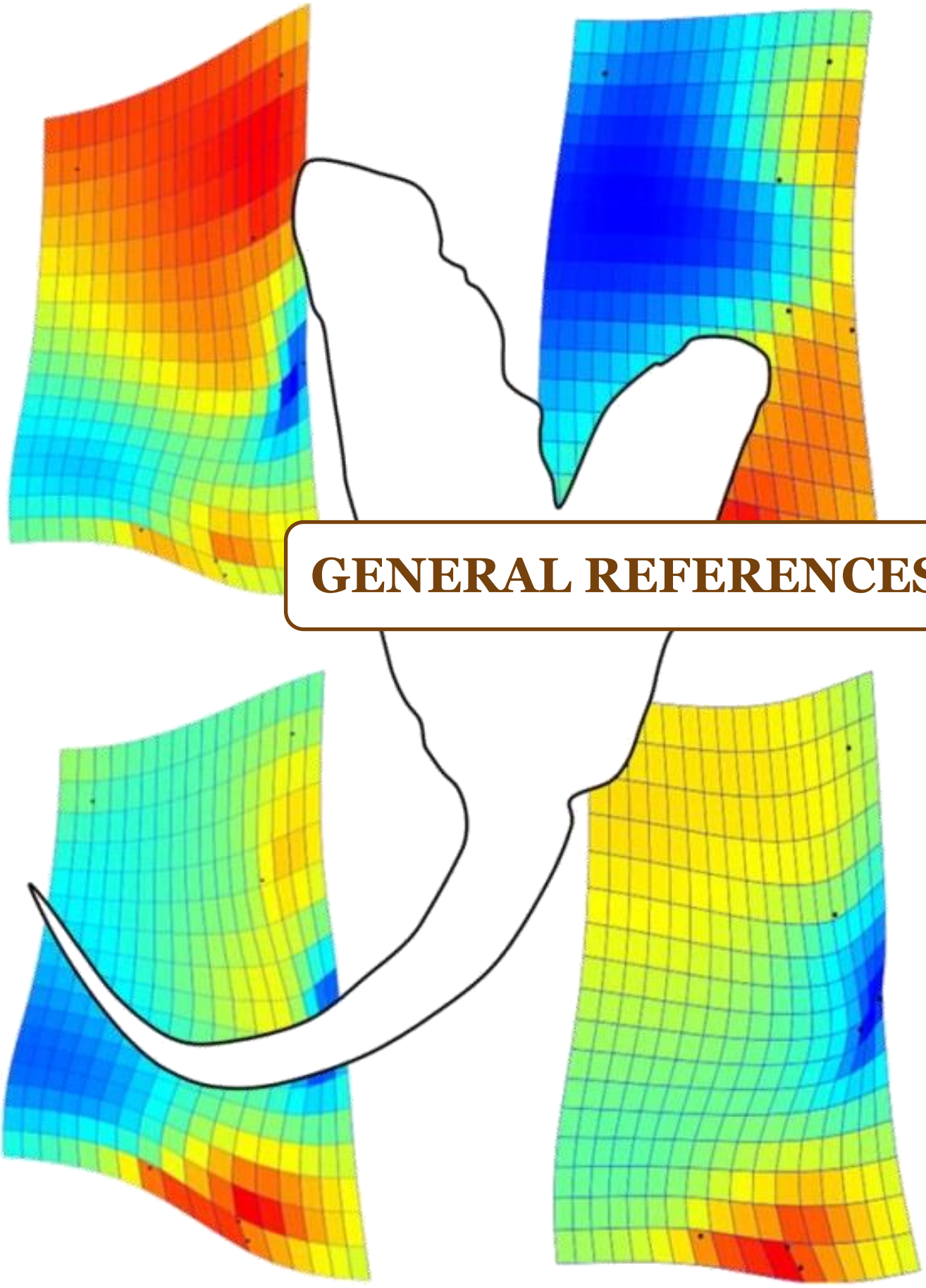
5. Shape variation in the anchors of *Ligophorus* spp. was concentrated in some modules. The complexity of microhabitats (gill arch, segment or area) provided by fish gills and adaptive responses by monogeneans may have facilitated the formation of distinct modules. Since evolutionary modularity was not always significant, convergent evolution could partly account for this pattern. However, phylogeny was the major determinant of the shape variation in the anchors.

6. The morphological integration at both levels in the modules detected was strong.

7. The muscular arrangement of the haptoral elements in the species of *Ligophorus* was consistent with the formation of functional modules for attachment to the gill and the evolutionary integration into the ventral and dorsal anchors.

8. Parasite phylogeny seems to play a major role in determining the shape and, to lesser degree, the size of the haptoral anchors in *Ligophorus* spp. In addition, no clear evidence for host-driven homoplasy in *Ligophorus* spp. was found.

9. Evaluating phylogenetic signal in morphological characters using geometric morphometric served to disentangle the roles of convergence and evolutionary history determining the morphology of anchors in *Ligophorus* spp.



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