UNIVERSIDAD DE CÁDIZ
FACULTAD DE CIENCIAS DEL MAR Y AMBIENTALES
DEPARTAMENTO DE BIOLOGÍA

¿Variabilidad amplia o diversidad oculta? Sistemática del Orden Runcinida (Gastropoda, Heterobranchia) como ejemplo de la necesidad de una reevaluación de la biodiversidad marina europea.

TESIS DOCTORAL
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¿VARIABILIDAD AMPLIA O DIVERSIDAD OCULTA? SISTEMÁTICA DEL ORDEN RUNCINIDA (GASTROPODA, HETEROBRANCHIA) COMO EJEMPLO DE LA NECESIDAD DE UNA REEVALUACIÓN DE LA BIODIVERSIDAD MARINA EUROPEA.

Memoria presentada por Ana Karla Araújo Moreira para optar al grado de Doctor por la Universidad de Cádiz

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CERTIFICAN:

Que la presente memoria titulada "¿Variabilidad amplia o diversidad oculta? Sistemática del Orden Runcinida (Gastropoda, Heterobranchia) como ejemplo de la necesidad de una reevaluación de la biodiversidad marina europea", presentada por Dña. Ana Karla Araujo Moreira, ha sido realizada bajo su dirección y autorizan su presentación y defensa para optar al Grado de Doctor por la Universidad de Cádiz.

Juan Lucas Cervera Currad

Ma Pa.

Fdo. Juan Lucas Cervera Currado

Fdo. Marta Pola Pérez

A mi FAMILIA

ÍNDICE

Todo tiene su tiempo,
y todo lo que se quiere debajo del cielo
tiene su hora
Eclesiastes 3:1

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Y aunque todo lo escrito aquí nunca sea suficiente para expresar toda mi gratitud, allá vamos...

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RESUMEN

El orden Runcinida (Heterobranchia, Euthyneura) está constituido por pequeñas babosas limaciformes que alcanzan un máximo de 8 mm de longitud. Tradicionalmente considerado un suborden dentro de Cephalaspidea, Runcinida fue elevado al rango de orden tras diversos estudios que demostraron que no formaba parte de la radiación de Cephalaspidea. La clasificación dentro de Runcinida también ha sufrido cambios en cuanto al número de familias y subfamilias que lo integran según los autores. Los caracteres morfológicos tradicionalmente utilizados para identificar y delimitar especies son insuficientes, ya que en muchos casos son muy semejantes e incluso las pequeñas variaciones plantean dudas de si serían variaciones intra o interespecíficas. Asimismo, la mayoría de las especies conocidas de runcínidos se encuentran en aguas europeas. Por lo tanto, el objetivo de la presente Tesis Doctoral es el estudio del orden Runcinida en Europa con el objetivo de evaluar su diversidad a partir de la realización de estudios filogenéticos. De este modo se podrá: 1) revisar la sistemática de las especies europeas, 2) detectar potenciales complejos de especies, así como definir criterios para delimitar sus especies, con la descripción de nuevos taxones si fuera necesario, 3) testar la validez de los caracteres diagnósticos tradicionales, 4) actualizar el conocimiento de la distribución geográfica de las especies estudiadas y 5) contribuir para la actualización de los inventarios de la fauna marina de diferentes países europeos. Para ello, se han llevado a cabo análisis filogenéticos basados en las secuencias parciales de dos marcadores mitocondriales (citocromo *c* oxidasa subunidad I y 16S rRNA) y uno nuclear (Histona 3). Además, se ha estudiado la morfología y anatomía interna de ejemplares procedentes del litoral atlántico europeo, incluyendo el mar Mediterráneo.

Los resultados obtenidos en este Tesis apoyan la monofilia del orden Runcinida y la del género *Runcina*, si se excluyen varios ejemplares de runcínidos incorrectamente atribuidos inicialmente a dicho género. Además, se han detectado cuatro complejos de especies bajo los nombres de *Runcina coronata, Runcina brenkoae, Runcina ferruginea* y *Runcina adriatica*, lo que ha permitido la descripción de 10 especies y un género nuevo para la Ciencia. Se confirma la presencia del género *Runcinida* en aguas europeas. Se rechaza el nombre *Pseudoilbia avellana*, rescatando el nombre original de la especie, *Runcina avellana*, así como se confirma la validez del nombre *Lapinura divae*. En relación a los caracteres morfológicos, estos resultaron ser muy poco informativos y de poca fiabilidad a la hora de separar especies, aunque pueden ser algo más informativos para diferenciar géneros. Finalmente, los resultados contribuyen a la actualización de los inventarios de la fauna marina de diferentes países europeos (Croacia, Italia, Francia, Portugal), y muy especialmente a la del "Inventario Español de Especies Marinas".

SUMMARY

The order Runcinida (Heterobranchia, Euthyneura) is composed of small limaciform sea slugs that can reach a maximum length of 8 mm. Traditionally considered a suborder within Cephalaspidea, Runcinida was included in its own Order after several studies that demonstrated that it was not part of the Cephalaspidea radiation. Classification within Runcinida has also changed in terms of number of families and subfamilies that comprised it according to various authors. Morphological characters traditionally used to identify and delimit species are insufficient, because in many cases they look alike and also because small variations have raised doubts as to whether they may refer to intraspecific variability or reflect distinct species. Most of the known species of runcinids are in European waters. Therefore, the aim of this PhD thesis is to study the order Runcinida in Europe in order to assess their diversity phylogenetic analyses. The specific objectives are: 1) to review the systematics of European species, 2) to detect potential species complexes, as well as define criteria to delimit their species, with the description of new taxa if necessary, 3) to test the validity of the traditional diagnostic characters, 4) to update knowledge about the geographical distribution of the species studied and 5) to contribute to the updating of marine fauna inventories in different European countries. For this, phylogenetic analyses based on partial sequences of two mitochondrial markers (cytochrome *c* oxidase subunit I and 16S rRNA) and a nuclear marker (Histone H3) were performed. In addition, the morphology and internal anatomy of specimens from the European Atlantic coast, including the Mediterranean Sea, were studied.

The results obtained in this thesis support the monophyly of the order Runcinida and the genus *Runcina*, after excluding several specimens initially incorrectly attributed to that genus. In addition, four species complex have been detected under the names *Runcina coronara, Runcina brenkoae, Runcina ferruginea* and *Runcina adriatica*, which has led to the description of 10 new species and a new genus. The presence of the genus *Runcinida* in European waters is confirmed. The name *Pseudoilbia avellana* is rejected, recovering the original name of the species, *Runcina avellana*, and the validity of the name *Lapinura divae* is also confirmed. Concerning the morphological characters, these resulted to be uninformative and unreliable to separate species, although they may be somewhat more informative to differentiate genera. Finally, the results contribute to updating the marine fauna inventories of different European countries (Croatia, Italy, France, Portugal) and, specially, the “Inventario Español de Especies Marinas”.

INTRODUCCIÓN GENERAL

Los Gasterópodos constituyen la clase más importante y amplia de los Moluscos, así como el taxón con mayor número de especies, y el más diverso morfológica y ecológicamente, con representación en los hábitats tanto marinos, como dulceacuícolas y terrestres (Aktipis *et al.*, 2008, Dinapoli y Klussmann-Kolb, 2010). Según Appeltans *et al.* (2012), las especies de gasterópodos marinos conocidas actualmente (entre 32.000 y 40.000) representan únicamente el 35% del total de las especies marinas de dicho taxón.

Tradicionalmente, la clase Gastropoda ha sido dividida en tres subclases: Prosobranchia, Opisthobranchia y Pulmonata (Milne-Edwards, 1848, Thiele, 1931, Franc, 1968a, b, c [in Grassé, 1968], Boss, 1982). Esta clasificación clásica de los gasterópodos aún está presente en algunos importantes libros de texto de Zoología (ej. Brusca y Brusca, 2005, Ruppert, Fox y Barnes, 2005, Hickman, 2009). Hickman *et al.* (2014) sigue usando los términos tradicionales, pero apunta que posiblemente sean grupos no monofiléticos. Brusca *et al.* (2017) ya incluye la clasificación moderna, mencionada a continuación.

El incremento en la realización de estudios filogenéticos ha conllevado grandes sorpresas relativas a las relaciones filogenéticas interfiléticas e intrafiléticas. Así, en la segunda mitad de los años 90, la monofilia de los Prosobranchia ya fué rechazada por análisis filogenéticos basados en datos morfológicos (Ponder y Lindberg, 1996, 1997). Sin embargo, ha sido el desarrollo y perfeccionamiento de las técnicas moleculares a finales de la década del siglo XX y en las dos primeras del siglo XXI, junto al desarrollo de potentes herramientas bioinformáticas, lo que ha permitido llevar a cabo análisis filogenéticos basados en marcadores moleculares (mitocondrales y nucleares) que han dado lugar a la formulación de hipótesis cada vez más robustas. Estas hipótesis han conducido a una verdadera "revolución" en la valoración de aquellos caracteres que tradicionalmente habían sido considerados como elementos rosbutos para el establecimiento de las clasificaciones. Esta "revolución" no sólo ha tenido lugar en el filo Moluscos, sino en el resto de filos de los Metazoos (ver Giribet, 2015, 2016). Así, en el caso de los Gasterópodos, diferentes análisis filogenéticos basados en marcadores moleculares han rechazado la monofilia no sólo de Prosobranchia, sino también de Opisthobranchia y Pulmonata (Atkipis *et al.*, 2008; Atkipis y Giribet, 2012; Stöger *et al.*, 2013, Zapata *et al.*, 2014; Uribe *et al.*, 2016), conduciendo a la actual clasificación de la clase Gastropoda en la que se reconocen seis subclases: Patellogastropoda, Neomphaliones, Vetigastropoda, Neritimorpha, Caenogastropoda y Heterobranchia (Bouchet *et al.*, 2017).

La subclase Heterobranchia Gray, 1840 es el grupo de gasterópodos más diverso ecológica y morfológicamente, incluyendo una gran variedad de especies con concha, si bien muchas han perdido su concha en el curso evolutivo (Aktipis *et al.*, 2008). Hace más de tres décadas, Haszprunar (1985) demostró que algunos grupos tradicionalmente considerados prosobranquios, estaban más relacionados con los Euthyneura (Opisthobranchia y Pulmonata), y redefinió el concepto de Heterobranchia que pasaría a incluir dos grupos: Euthyneura y el grupo informal denominado "Lower Heterobranchia" (heterobranquios basales antes considerados como prosobranquios) (Haszprunar, 1985; Mordan y Wade, 2008; Wägele *et al.*, 2008).

La monofilia de Heterobranchia está bien sustentada por diversas sinapomorfias, como son: la concha larval levógira, la ausencia de un verdadero ctenidio y de verdaderos cartílagos odontofóricos, el hermafroditismo, un esófago simple, y distintos caracteres a nivel ultraestructural en el esperma, entre otras (Haszprunar, 1985; Ponder y Lindberg, 1997; Wägele *et al.* 2014). Sin embargo, en las últimas décadas, muchos estudios moleculares (Grande *et al.*, 2004; Dinapoli y Klussmann-Kolb, 2010; Jörger *et al.*, 2010; Kocot *et al.*, 2013, Wägele *et al.*, 2014) han demostrado que los grupos que constituyen el taxón Heterobranchia (Opisthobranchia y Pulmonata, además de los heterobranquios basales) no son monofiléticos. Estos estudios moleculares han contribuido, además, a la actualización de la clasificación de los heterobranquios.

De este modo, una nueva clasificación fue propuesta por Jörger *et al.* (2010) a partir de datos moleculares, la cual divide a la subclase Heterobranchia en "**Lower Heterobranchia**" (incluyendo Acteonoidea y excluyendo Pyramidelloidea y Glacidorboidea) y **Euthyneura**, subdivididos en tres grupos: Nudipleura (Nudibranchia y Pleurobranchida), Euopisthobranchia (Umbraculida, Aplysiida, Runcinida, Pteropoda y Cephalaspidea *s.s.*) y Panpulmonata (incluye todos los antiguos "pulmonados", los antiguos grupos de "opistobranquios" Sacoglossa y Acochlidimorpha, y los Pyramidelloidea y Glacidorboidea, previamente considerados heterobranquios basales). Schrödl *et al.* (2011) propusieron el nombre Tectipleura para el clado integrado por los grupos Euopisthobranchia y Panpulmonata, siendo dicho clado grupo hermano de Nudipleura. Kano *et al.* (2016) propusieron una nueva modificación a la clasificación de Jorgër *et al.* (2010) al incluir en su estudio el grupo Ringiculimorpha. De este modo, la nueva división de Heterobranchia incluye "**Lower Heterobranchia**" (excluyendo Acteonoidea) y **Euthyneura** subdivididos en tres nuevos grupos: Acteonimorpha (Rissoelloidea y Acteonoidea), Ringipleura (Ringiculimorpha y Nudipleura) y Tectipleura (Euopisthobranchia y Panpulmonata) (Fig. 1).

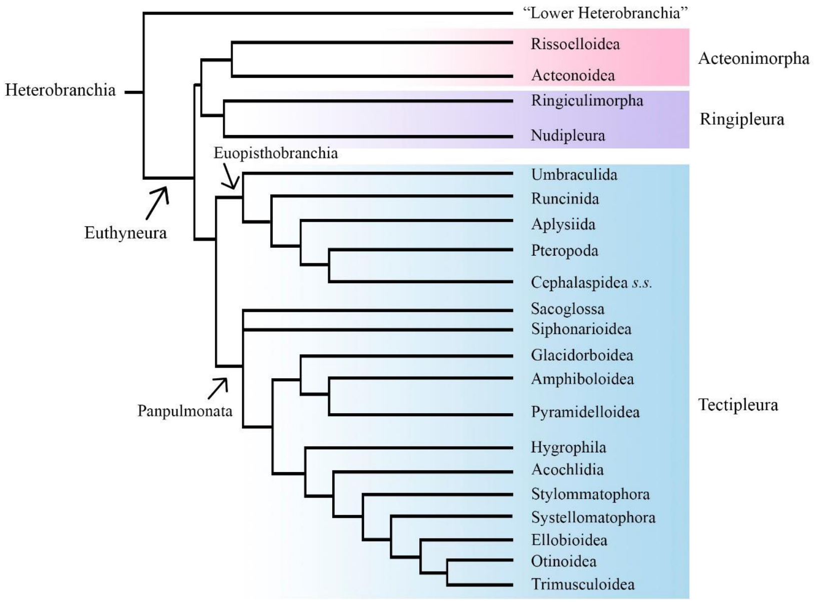

Figura 1. Clasificación actual de Heterobranchia (Basado en Jörger *et al*., 2010, Wägele *et al*., 2014 y Kano *et al*., 2016).

Orden Runcinida

Dentro de Euopisthobranchia, el orden Runcinida Burn, 1963 – según la categoría taxonómica y la terminología asignada por Bouchet *et al*. (2017) – está formado por pequeñas babosas marinas que alcanzan un máximo de 8 mm de longitud (Baba, 1937). Habitan costas rocosas intermareales o poco profundas, y son herbívoros especializados que se alimentan de macroalgas (Burn, 1963; Thompson y Brodie, 1988; Schmekel y Cappellato, 2001). Actualmente, el orden cuenta con un total de 61 especies descritas distribuidas entre los océanos Atlántico (incluyendo el Mar Mediterráneo) y Pacífico (MolluscaBase, 2020).

A lo largo de los años, la clasificación dentro del orden Runcinida ha sufrido algunos cambios; en función del autor, el número de familias y subfamilias ha variado entre una o dos familias y ninguna o dos subfamilias (Tabla 1). Así, Odhner (1924), Baba (1967) y Gosliner (1991), por ejemplo, consideraban que todos los géneros descritos pertenecían a una única familia, Runcinidae. Sin embargo, Burn (1963) estableció una segunda familia, Ilbiidae, en donde estarían reunidos los géneros que presentan una concha externa. Además, también propuso la división de la familia Runcinidae en dos subfamilias: Runcinidae y Ilbiinae. Actualmente, el orden Runcinida está integrado por dos familias: Ilbiidae Burn, 1963 y Runcinidae H. Adams y A. Adams, 1854, esta última sin subfamilias. Esa clasificación fue introducida por Burn y Thompson (1998), en la que la familia Ilbiidae incluía tres géneros (*Ilbia* Burn, 1963, *Pseudoilbia* Miller y Rudman, 1968 y, posteriormente, *Fofinha* Moro y Ortea, 2015) y la familia Runcinidae ocho géneros (*Runcina* Forbes [in Forbes and Hanley], 1851, *Ildica* Bergh, 1889, *Runcinella* Odhner, 1924, *Runcinida* Burn, 1963, *Metaruncina* Baba, 1967, *Lapinura* Er. Marcus and Ev. Marcus, 1970, *Edmundsina* Ortea, 2013 and *Karukerina* Ortea, 2013). Sin embargo, Spencer *et al*. (2011) consideran el género *Pseudoilbia* como parte de Runcinidae y, por lo tanto, según estos autores y MolluscaBase (2020) esta familia está formada por nueve géneros e Ilbiidae por tan solo dos géneros. En esta Memoria, seguiremos la clasificación de Burn y Thompson (1998), Ortea *et al*. (2013), Burn (2015), y Moro y Ortea (2015), los cuales consideran *Pseudoilbia* como parte de la familia Ilbiidae, ya que la inclusión de *Pseudoilbia* en Runcinidae se realizó sin ningún tipo de base o argumento.

La familia Ilbiidae está caracterizada, principalmente, por la ausencia de branquia, concha y placas gástricas (Burn, 1963, Miller y Rudman, 1968). La familia Runcinidae, la más numerosa, se caracteriza por la presencia de una concha interna rudimentaria en ocho especies, si bien en otras está ausente, rádula con diente raquídeo bilobulado y posesión de una branquia (hasta 4 ramas branquiales) en la parte posterior del cuerpo. La branquia puede ubicarse a la derecha del ano, dispuesta en semicírculo, o rodeando el ano (Burn, 1963, Schmekel y Cappellato, 2001).

En general, los runcínidos se caracterizan principalmente por presentar un manto continuo, un pie no dividido, ausencia de parapodios o extensiones laterales y por la presencia de cuatro placas gástricas quitinosas en el molino gástrico. Además, pueden presentar una pequeña concha externa (*Ildica* y *Lapinura*) o una concha interna rudimentaria (*Metaruncina*, *Karukerina* y algunas especies de *Runcinella* y *Runcina*), aunque está ausente en gran parte de las especies del grupo. Mayoritariamente presentan una pequeña branquia, bien alrededor o a la derecha del ano, pero ésta también puede estar ausente como ocurre en los géneros *Ilbia* y *Pseudoilbia*. El ano es terminal y se abre justo a la derecha de la línea media del cuerpo. No presentan tentáculos cefálicos. Los tentáculos orales están presentes solamente en el género *Ildica*. Las mandíbulas y la rádula están presentes. Las hileras de los dientes radulares están formada por un diente raquídeo (uni, bi o tricúspide) y uno o dos dientes laterales (lisos o denticulados) respecto del raquídeo. Las placas gástricas están presentes en todos los runcínidos, excepto en el género *Pseudoilbia*. El orificio genital común se abre posteriormente en el lado derecho, mientras que el poro del órgano copulador masculino se abre anteriormente a la derecha de la apertura de la boca (Burn, 1963; Ghiselin, 1963; Miller y Rudman, 1968; Thompson, 1976; Kress, 1977; Cervera *et al.*, 1991; Burn y Thompson, 1998; Schmekel y Cappellato, 2001; Moro y Ortea, 2015) (Figs 2 y 3).

Tabla 1. Clasificación dentro del orden Runcinida en función del autor.

Autores	Familia(s)	Subfamilias	Generos
Odhner (1924), Baba (1937), Pruvot-Fol (1953, 1954), Ev. Marcus y Er. Marcus (1963), Ghiselin (1963), Baba (1967), Powell (1979), Walsby y Ballantine (1984), Gosliner (1991)	Runcinidae	-	*Runcina, Runcinida, Runcinella, Ildica, Pseudoilbia, Ilbia, Metaruncina, Lapinura*
Burn (1963, 1966), Er. Marcus y Ev. Marcus (1970), Boss (1982), Vaught (1989), Hoff y Carlson (1990)	Runcinidae	Rucininae	*Runcina, Ildica, Runcinella, Runcinida, Metaruncina, Lapinura*
		Ilbiinae	*Ilbia, Pseudoilbia*
	Ildicidae	-	*Ildica, Lapinura*
Thompson y Brodie, (1988), Schmekel y Cappellato (2001, 2002)	Runcinidae	-	*Runcina, Runcinida, Runcinella, Metaruncina, Ilbia, Pseudoilbia*
	Ildicidae	-	*Ildica, Lapinura,*
Burn y Thompson (1998) Ortea *et al*. (2013), Burn (2015), Moro y Ortea (2015)	Runcinidae	-	*Runcina, Ildica, Runcinida, Runcinella, Metaruncina, Lapinura, Edmundsina, Karukerina*
	Ilbidae	-	*Ilbia, Pseudoilbia, Fofinha*
Spencer *et al*., 2011; MolluscaBase, 2020	Runcinidae	-	*Runcina, Ildica, Runcinida, Runcinella, Metaruncina, Pseudoilbia, Lapinura, Edmundsina, Karukerina*
	Ilbidae	-	*Ilbia, Fofinha*

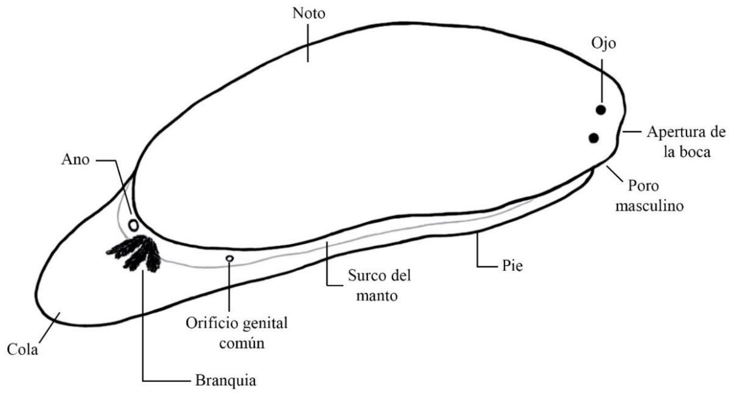

Figura 2. Esquema general de la anatomía de los runcínidos. Ilustración de Ana Karla Araujo Moreira.

En los primeros años del presente siglo, estudios filogenéticos basados en datos morfológicos (Dayrat y Tillier, 2002; Wägele y Klussmann-Kolb, 2005) y moleculares (Dayrat *et al*., 2001; Grande *et al*., 2004a, b; Vonnemann *et al*., 2005) llegaron a resultados poco concluyentes en cuanto a la relación entre Runcinida y Cephalaspidea, lo que volvió a cuestionar la inclusión de Runcinida dentro de Cephalaspidea. Si bien, las principales características de Runcinida (manto continuo, pie no dividido, ausencia de parapodios/extensiones laterales, presencia de cuatro placas gástricas) son bastante diferentes respecto a los Cephalaspidea s.s.. El sistema reproductor, típico de Cephalaspidea, ha sido uno de los principales caracteres utilizados para retener Runcinida (=Peltacea Odhner, 1939; =Runcinacea Burn, 1963) con el rango de suborden, dentro de Cephalaspidea (Odhner, 1939; Burn, 1963; Schmekel, 1985). No obstante, Colosi (1915, 1921) fue el primer autor en cuestionar la ubicación de Runcinida dentro de Cephalaspidea. Décadas después, Odhner (in Franc, 1968) sugirió que Runcinida debía ser considerado con el rango de Orden. Aun así, a lo largo de los años, muchos autores han seguido considerando Runcinida como parte de Cephalaspidea (Burn, 1963; Ghiselin, 1963, 1965; Baba, 1967; Er. Marcus y Ev. Marcus, 1970; Kress, 1977; Schmekel, 1985; Thompson y Brodie, 1988; Gosliner, 1990, 1991; Cervera *et al*., 1991; Mikkelsen, 1993, 1996; Schmekel y Cappellato, 2001, 2002).

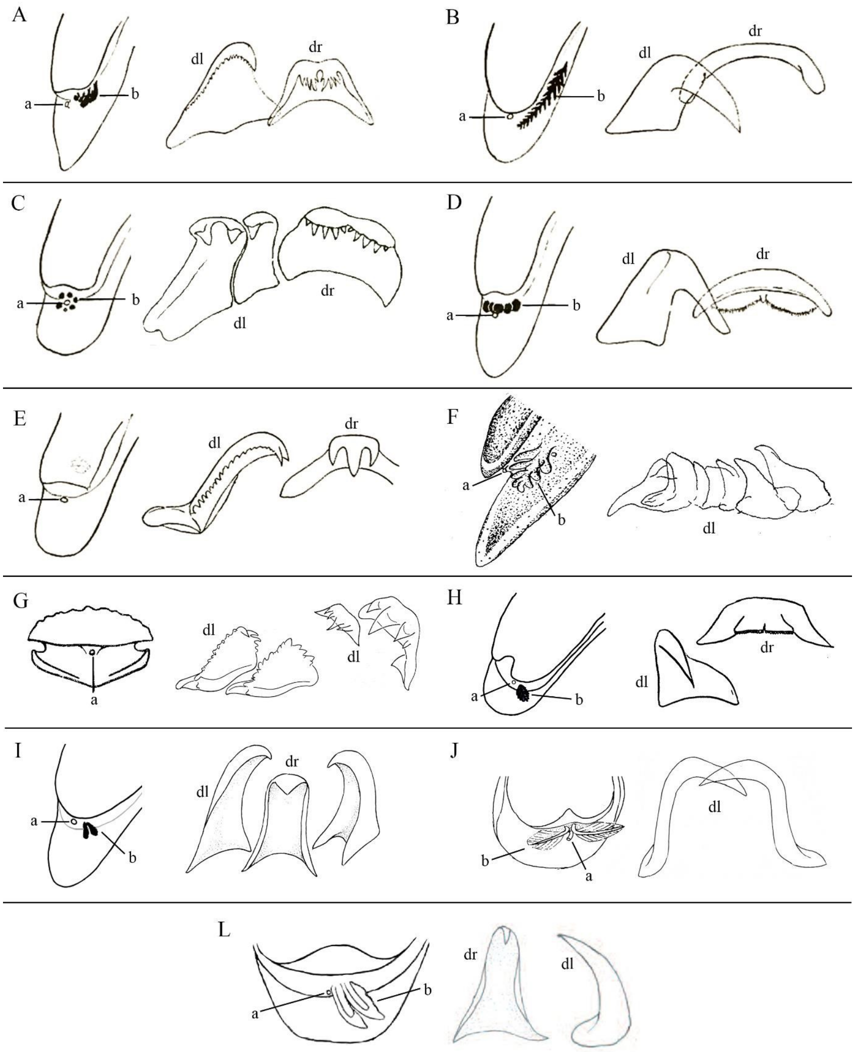

Figura 3. Esquema de las posiciones del ano y branquia, y diversidad de los dientes radulares en los géneros del orden Runcinida. **A.** *Runcina.* **B.** *Ildica.* **C.** *Runcinella.* **D.** *Runcinida.* **E.** *Ilbia.* **F.** *Metaruncina.* **G.** *Pseudoilbia.* **H.** *Lapinura.* **I.** *Edmundsina.* **J.** *Karukerina.* **L.** *Fofinha.* Adaptado de Burn (1963) (A, B, C, D, E), Baba (1967) (F), Miller y Rudman (1968) (G), Er. Marcus y Ev. Marcus (1970) (H), Ortea (2013) (I), Ortea et al. (2013) (J) y Moro y Ortea (2015) (L). Abreviaciones: a, ano; b, branquia; dl, diente lateral; dr, diente raquídeo.

Sin embargo, no fue hasta finales de la primera década de siglo XXI cuando Malaquias *et al.* (2009), a partir de detallados estudios filogenéticos basados en marcadores moleculares demostraron que los runcínidos no formaban parte de la radiación de Cephalaspidea, lo que podría justificar que se les asignase el rango de Orden, como ya propuso Odhner (in Franc, 1968). Las conclusiones de estos autores han sido corroboradas por otros estudios filogenéticos moleculares posteriores (Jörger *et al*., 2010; Göbbeler y Klussmann-Kolb, 2011; Wägele *et al*., 2014; Oskars *et al*., 2015).

El pequeño tamaño de los integrantes de este grupo y el hecho de que la mayoría de las especies tienen patrones de colores crípticos oscuros y apagados, hace que los runcínidos sean difíciles de detectar e identificar (Gosliner, 1990, Ballesteros *et al*., 2016, Prkić *et al*., 2018). Además, tras el análisis de la bibliografía, los caracteres morfológicos tradicionalmente utilizados para identificar y delimitar especies, así como para definir géneros o familias no son realmente suficientes ya que en muchos casos dichos caracteres son muy semejantes o las pequeñas variaciones que se observan entre individuos plantean la duda de si son variaciones intraespecíficas o interespecíficas (Schmekel y Cappellato, 2001, 2002, Ortea *et al*., 2013, Araujo *et al*., 2019 [ver Capítulo II]).

De las 61 especies del orden Runcinida, la mayoría de las especies de runcínidos descritas se concentran en aguas europeas - Océano Atlántico, incluido el Mar Mediterráneo - y pertenecen a los géneros *Runcina*, *Pseudoilbia* y *Runcinella*. Entre ellos, *Runcina* es el género con el mayor número de especies (31 especies) presentes en Europa. *Pseudoilbia* y *Runcinella* poseen tan solo una especie cada género descrita para aguas europeas (Cervera *et al*., 2004, Ortea *et al*., 2015, Ballesteros *et al*., 2016, MolluscaBase, 2020).

Objetivos:

El objetivo de este estudio es contribuir al conocimiento de la biodiversidad marina europea a través de la realización de un estudio monográfico de la sistemática del orden Runcinida (Gastropoda, Heterobranchia). De forma específica, se persiguen los siguientes objetivos:

1. Revisar la sistemática de las especies europeas del orden Runcinida.
2. Detección de potenciales complejos de especies en el orden Runcinida, en el ámbito de las costas europeas.
3. Definir los criterios para delimitar las especies en los complejos de especies detectados.
4. Testar la validez de los caracteres diagnósticos tradicionales utilizados en el grupo.
5. Describir, si fuera necesario, nuevos taxones resultantes de la detección de especies crípticas o pseudocrípticas para las que los nombres específicos ya disponibles no puedan ser aplicables.
6. Actualizar el conocimiento de la distribución geográfica de las especies estudiadas.
7. Contribuir a la actualización de los inventarios de la fauna marina de diferentes países europeos, con especial atención al “Inventario Español de Especies Marinas”.

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RESULTADOS

Capítulo 1

Molecular phylogeny of European Runcinida (Gastropoda, Heterobranchia): the discover of an unexpected pool of complex species, with special reference to the case of *Runcina coronata* (Quatrefages, 1844)

Declaración: Esta obra no se considera publicada a los efectos previstos en el Art. 8.2 del Código Internacional de Nomenclatura Zoológica. No constituye, por tanto, un registro científico público y permanente para fines de nomenclatura zoológica.

Molecular phylogeny of European Runcinida (Gastropoda, Heterobranchia): the discover of an unexpected pool of complex species, with special reference to the case of *Runcina coronata* (Quatrefages, 1844)

ABSTRACT

Runcinida is a small heterobranch order of sea slugs with 61 known species distributed in Atlantic and Pacific Oceans. Constituted by two families, Ilbiidae and Runcinidae, its taxonomical status has been discussed for a long time until several recent molecular studies supported its ordinal assignment. However, little is known about the phylogenetic relationships within Runcinida. Since most of described runcinids species are concentrated in European waters, the first molecular phylogeny of European runcinids is presented here. We also investigate the taxonomic status of the type species *Runcina coronata* (Quatrefages, 1844), due to an apparent confusion that surrounds it. For this study we follow an integrative approach based on new sequences of two mitochondrial genes (COI and 16S) and one nuclear gene (H3), and morpho-anatomical characters. Our results suggest the monophyly of Runcinida and reveal *Runcina coronata* as a species complex. Therefore, we here describe three new species (*R. caletae* sp. nov., *R. flavonigra* sp. nov. and *R. tingitana* sp. nov.) and redescribe *R. coronata* properly.

INTRODUCTION

The order Runcinida Burn, 1963 – according to taxonomic category and terminology assigned by Bouchet *et al*. (2017) – is composed by small heterobranch sea slugs that can reach a maximum length of 8 mm. These sea slugs are specialized herbivores, feeding on macrophytic algae, and inhabiting intertidal and shallow rocky shores (Burn, 1963, Thompson and Brodie, 1988). All members are mostly characterized by having a dorsum not divided transversely into two shields – like in the cephalaspideans–, an undivided foot, a small gill next to the anus; lack of lateral extensions or parapodia, presence of four gizzard plates and an external seminal groove (Burn, 1963, Thompson, 1976, Burn and Thompson, 1998).

Currently containing 61 described species distributed among Atlantic (including Mediterranean Sea) and Indo-Pacific Oceans, the order Runcinida is constituted by two families: Ilbiidae Burn, 1963 and Runcinidae H. Adams & A. Adams, 1854. Ilbiidae is composed by three genera: *Ilbia* Burn, 1963 (two species), *Pseudoilbia* Miller and Rudman, 1968 (two species), and *Fofinha* Moro and Ortea, 2015 (one species). Members of this family are characterized by the absence of shell, gills and/or gizzard plates, and presence of a pedal furrow. However, none of the genera present all these characteristics, for example, *Ilbia* and *Pseudoilbia* share the absence of the gill and the presence of pedal furrow, while *Pseudoilbia* and *Fofinha* share the absence of gizzard plates. Concerning the radula, it differs radically among the genera. Thus, *Ilbia* has triseriate (1.1.1) radula with tricuspid rachidian teeth; that of *Pseudoilbia* (2.0.2) is tetraseriate lacking rachidian tooth and with asymmetrical lateral teeth; and *Fofinha* has a biseriate (0.1.1) radula with unicuspid rachidian teeth. The family Runcinidae includes eight genera: *Runcina* Forbes [in Forbes and Hanley], 1853 (40 species), *Ildica* Bergh, 1889 (one species), *Runcinella* Odhner, 1924 (three species), *Runcinida* Burn, 1963 (three species), *Metaruncina* Baba, 1967 (two species), *Lapinura* Er. Marcus and Ev. Marcus, 1970 (three species), *Edmundsina* Ortea, 2013 (three species) and *Karukerina* Ortea, 2013 (one species). Members of this family are characterized by having a radula with rachidian tooth bilobed, presence of gills – up to four pinnulae – and four gizzard plates. The shell can be present or absent.

Early described by Odhner (1939) as Peltacea and subsequently named as Runcinacea by Burn, 1963, Runcinida was considered as a suborder – the most "derived" – within Cephalaspidea only by having in common the presence of the external seminal groove (Odhner, *op.cit*., Burn, 1963, Schmekel, 1985). Colosi (1915, 1921) was the first to challenge the position of Runcinida within Cephalaspidea. Decades later, Odhner (in Franc, 1968) suggested that Runcinida should be ranked as order. Even so, over the years, many authors have continued to consider Runcinida as part of Cephalaspidea (Burn, 1963, Ghiselin, 1963, Baba, 1967, Er. Marcus and Ev. Marcus, 1970, Kress, 1977, Schmekel, 1985, Thompson and Brodie, 1988, Vaught, 1989, Gosliner, 1990; 1991, Cervera *et al*., 1991, Mikkelsen, 1993; 1996, Millard, 1997, Schmekel and Cappellato, 2001; 2002).

Several phylogenetic studies on Heterobranchia based on morphological (Dayrat and Tillier, 2002, Wägele and Klussmann-Kolb, 2005) and molecular data (Dayrat *et al*., 2001; Grande *et al*., 2004a, b; Vonnemann *et al*., 2005) questioned the possible inclusion of Runcinida in the Cephalaspidea, but the results were alwayas inconclusive due to lack of resolution of the various phylogenetic hypotheses. Malaquias *et al*. (2009) based on molecular phylogenetics analysis, produced the first sound evidence for the inclusion of runcinids in their own Order as early proposed by Odhner (in Franc, 1968) and later supported by molecular phylogenetics by Jörger *et al*. (2010), Wägele *et al*. (2014) and Oskars *et al*. (2015).

Despite the progresses on the phylogenetic position of runcinids among the tree of life of the Heterobranchia, little is still known about the phylogenetic relationships within Runcinida. Burn (1963) was the first to discuss the relationships among members of Runcinida based on morphological data after describing the genus *Ilbia* which he suggested to be the most derived due to the presence of very different characters compared to others runcinids. Later, Baba (1967) proposed a re-arrangement of the genera within Runcinida based mainly on their gill features. However, this re-arrangement did not gain enough acceptance to substitute the classification proposed by Burn (*op. cit.*).

The small size of the runcinids and the fact that most species have dark cryptic colour pattern, makes them hard to detect and identify (Gosliner, 1990, Ballesteros *et al*., 2016, Prkić *et al.*, 2018). Besides, morphological characters traditionally used on other sea slugs to identify and delimit species as well as to define genera and/or families are not sufficient because most species look alike or extremely different, as in the case of the families Ilbiidae and Runcinidae. In addition, small variations observed between specimens have raised doubts as to whether they may refer to intraspecific variability or reflect already distinct species. Nonetheless new species and genera have been described in the last years (Ortea, Moro and Bacallado, 2013, Ortea *et al*., 2013, Ortea *et al*., 2014; Ortea and Moro, 2015; Araujo *et al.*, 2019 [see Chapter II]).

Most of the described runcinids species are concentrated in European waters – Atlantic Ocean including the Mediterranean Sea – and belong to the genera *Runcina*, *Pseudoilbia* and *Runcinella*. Among them, *Runcina* is the genus with the largest number of species (31 species) present in Europe. Its type species, *Runcina coronata* (Quatrefages, 1844), is reported from England to the Mediterranean coastline of France (Quatrefages, 1844, Alder and Hancock, 1846, Vayssière, 1883, Schmekel and Cappellato, 2002, Cervera *et al*. 2004, Ballesteros *et al*., 2016). This species is characterized by an almost elliptical body with the head zone bilobed and the posterior part of the body rounded. The notum has brown colour, which becomes much lighter at the edges. It also has very characteristic small dull white pearls forming a semicircle on the head zone posterior to eyes and on the end of the notum. Three small and slightly pinnate gills are situated on the right side of the anus (Quatrefages, 1844, Alder and Hancock, 1846, Forbes and Hanley, 1851).

Vayssière (1883) reported *R. coronata* from Marseille (French Mediterranean coast), although some characteristics of his specimens differed from the original description of *R. coronata*. Burn (1963) used some of these differences (shape of the body, colouration, number of crests of the gizzard plates) to propose that Mediterranean *R. coronata* was a different species. In fact, he suggested that Colosi (1915) re-described these specimens from the Mediterranean as *R. calaritana*. Nevertheless, Pruvot-Fol (1954) had already suggested *R. calaritana* as synonym of the Mediterranean *R. coronata*. Moreover, Pruvot-Fol (*op. cit.*) also mentioned *R. capreensis* Mazzarelli (1893) as another possible synonym of the Mediterranean

R. coronata. Another species, *Runcina aurata* García, López, Luque and Cervera, 1986, from southern Atlantic coast of Spain, was described based on several differences with respect to the specimens identified as *R. coronata* that the authors collected in the Strait of Gibraltar (García *et al.*, 1986). Later, Cervera *et al*. (1991) synonymized *R. aurata* with *R. coronata* based on a detailed appraisal of the original description of *R. coronata*, leading them to conclude that the specimens attributed to *R. aurata* belonged to *R. coronata* and that the specimens attributed to *R. coronata* by García *et al*. (1986) could be *Runcina ornata* (Quatrefages, 1844) due to the similarity of the external features.

R. coronata was described as *Pelta coronata* by Quatrefages (1844) with specimens from Brehat, Bretagne (Atlantic coastline of France) together with *Runcina ornata* (=*Pelta ornata*), also by the same author and from the same locality. Two years later, Alder and Hancock (1846) found some specimens from Torbay (southern of England) that were very similar to those described by Quatrefages and also to *Limapontia nigra* Johnston, 1835, but differing from the former by the presence of the gills. Quatrefages (*op. cit*) does not observed gills in his specimens of *R. coronata*, which Alder and Hancock believed to be an erroneous observation. Nevertheless, these authors did not designate any specific name for these specimens. Years later, Forbes (1853) based on additional specimens also collected in Torbay, described the species *Runcina hancocki* which most remarkable difference respect to *R. coronata* was the presence of the gills. Vayssière (1883) in his "Monographie du *Pelta*" considered *Runcina* as synonym of *Pelta*. However, in 1951, the generic name *Pelta* Quatrefages, 1844 was suppressed and only the generic name *Runcina* Forbes [in Forbes and Hanley, 1851] was considered as a valid name (Lemche, 1967, Opinion n. 811).

The European species *Runcina coronata* has been subject of significant discussions as stated aboved. Unlike *R. coronata*, for the other species of European runcinids, there has been almost no taxonomic controversy, except in the case of *R. ferruginea* versus *R. zavodniki* (Thompson and Brodie, 1988, Schmekel and Cappellato, 2002).

Therefore, the primary objective of this study is to know the diversity and systematics of the European species of runcinids based on molecular phylogeny, although representatives of others localities outside of Europe have also been included. Secondly, due to the apparent confusion that surrounds the type species *Runcina coronata* (Quatrefages, 1844) we investigate here its taxonomic status. For this, we follow an integrative approach combining multi-locus molecular phylogenetics and morpho-anatomical characters of specimens from the southern England (Swanage), southwestern Spain (Cádiz, Andalusia) and, also include specimens from northwestern Morocco (Tangier).

MATERIALS AND METHODS

Taxon Sampling

The studied material was obtained from fieldwork conducted by the authors and colleagues, and through the study of museum collections. Voucher specimens are held in the collections of the Zoologische Staatssammlung München, ZSM (Munich, Germany), Museum Victoria, MV (Melbourne, Australia), Museu de Zoologia da Universidade de São Paulo, MZSP (São Paulo, Brazil), University Museum of Bergen, ZMBN (Bergen, Norway) and Museo Nacional de Ciencias Naturales (Madrid, Spain). The request to examine the material deposited in the Museo de Naturaleza y Arqueología de Santa Cruz de Tenerife (Canary Islands, Spain) was denied. In any case, due to the preservation method the material was not viable for molecular studies. Therefore, the species described for the Canary Islands could not be studied or included in this study.

For the molecular analyses 77 specimens of the order Runcinida were included, representing five genera: *Runcina* (71 specimens), *Metaruncina* Baba, 1967 (1 specimen), *Lapinura* Er. Marcus and Ev. Marcos, 1970 (2 specimens), *Ilbia* Burn, 1963 (1 specimen), *Runcinida* Burn, 1963 (1 specimen). The remaining two specimens were referred as "runcinid" sp. We also included the aplysiid *Aplysia dactylomela* Rang, 1828 and the acteonoid *Micromelo undatus* (Bruguière, 1792), which was used to root the tree as outgroup taxon. In total, 189 novel sequences were generated during the present work and 91 were obtained from GenBank (Table 1).

DNA extraction, amplification and sequencing

Tissue samples were taken from the foot and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Partial sequences of the mitochondrial *cytochrome c oxidase subunit I* (COI), and *16s ribosomal rRNA* (16S), and nuclear *histone-3* (H3) genes were amplified by polymerase chain reaction (PCR) using the universal primers: LCO1490 and HCO2198 (Folmer *et al*., 1994 for COI); 16S ar-L and 16br-H (Palumbi *et al*., 1991 for 16S); and H3aF and H3aR (Colgan *et al*., 1998 for H3). PCRs were conducted in a 25 µl reaction volume containing 1 µl of both forward and reverse primers (10 µM), 2.5 µl of dNTP (2 mM), a gene-dependent amount of magnesium chloride (25 mM), 0.25 µl of Qiagen DNA polymerase (5 units/µl), 5 µl of "Q-solution" (5x), 2.5 µl of Qiagen buffer (10x) (Qiagen Taq PCR Core Kit) and 2 µl of genomic DNA. Amplification of COI was performed with an initial denaturation for 5 min at 94ºC, followed by 35–36 cycles of 1 min at 94ºC, 30s at 45ºC (annealing temperature) and 1 min at 72ºC, with a final extension of 10 min at 72ºC. The 16S amplification began with an initial denaturation for 5 min at 94ºC, followed by 35–36 cycles of

1 min at 94ºC, 30s at 42 and 49ºC (annealing temperatures) and 1 min at 72ºC, with a final extension of 10 min at 72ºC. Amplification of H3 was performed with an initial denaturation for 5 min at 94ºC, followed by 35 cycles of 1 min at 94ºC, 30s at 52ºC (annealing temperature) and 1 min at 72ºC, with a final extension of 10 min at 72ºC. Successful PCR products were sent to Macrogen, Inc for purification and sequencing on a 3730XL DNA sequencer (Applied Biosystems).

Phylogenetic analyses

Sequences were edited in Genious v10.2.3 (Drummond *et al*. 2009) and aligned using MAFFT (Katoh *et al*., 2009) implemented in Geneious v10.2.3 (Drummond *et al*. 2009) with the default settings. Sequences from the protein-coding genes COI and H3 were translated into amino acids to check for stop-codons. Hypervariable regions of the 16S alignment where homology could not be confidently established were removed using Gblocks under relaxed settings (Tavalera and Castresana, 2007). Nevertheless, analyses including and excluding these regions provided similar results. Therefore, final analyses were performed including all bases. Sequences of the COI, 16S and H3 genes were trimmed to 658, 489 and 328 nucleotides, respectively. Single gene and concatenated (H3+COI+16S) analyses were performed. Saturation for the first, second and third codon positions of the COI and H3 genes were calculated in MEGA v7.0 (Kumar *et al*., 2016).

The best-fit evolutionary model for each gene was determined in jModeltest v2.1.6 (Guindon and Gascuel, 2003; Darriba *et al*., 2012), under the Akaike information criterion (AIC) (Akaike, 1974). The GTR + G + I model was selected for the COI and 16S genes, and K80 + G for the H3 gene. Bayesian inference (BI) analyses were performed in MrBayes v. 3.2.1 (Ronquist and Huelsenbeck, 2003) with random starting trees and two parallel runs of 10^7 generations. The models implemented were those estimated with jModeltest v2.1.6. The combined dataset was partitioned among genes and the “unlink” command was used to allow all parameters to vary independently within each partition. Convergence was checked in TRACER v1.7.1 (Rambaut *et al*., 2018) with a burn-in of 25%. Nodes with a posterior probability (PP) $\geq$ 0.95 (Alfaro, Zoller and Lutzoni, 2003) were considered well supported and discussed. Maximum likelihood (ML) analysis was executed using RAxML v8 (Stamatakis, 2014) and node support was assessed with nonparametric bootstrapping (BS) with 5000 replicates. Nodes with bootstrap values (BS) $\geq$ 70 (Hillis and Bull, 1993) were considered significant and were discussed. Both BI and ML trees were visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Species delimitation

For species delimitation analyses, Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al*., 2012), Bayesian Poisson Tree Processes (bPTP) (Zhang *et al*., 2013) and pairwise uncorrected *p*-distances were carried out. For the ABGD analyses we used the fast-evolving COI gene using the ingroup sequences obtained in this study and those available in Genbank. We used the default setting ($P_{\text{min}} = 0.001$, $P_{\text{max}} = 0.1$, Steps = 10, $X = 1.2$, Nb bins = 20) under the three models of evolution, namely Jukes-Cantor (JC69), Kimura (K80) and Simple Distance. The bPTP analysis is an updated version of the original maximum likelihood PTP (modelling speciation in terms of the number of substitutions), which adds Bayesian support values to delimit species. bPTP analyses were run with default parameters using the COI trees at the webserver (https://species.h-its.org/ptp/) (Zhang *et al*., 2013). Minimum and maximum pairwise uncorrected *p*-distances of COI within and between species were calculated in MEGA v7.0 using all sequences available (Kumar *et al*., 2016).

Morphology

In general, the external morphology of the specimens used for molecular analysis was previously examined from photographs of living animals and from laboratory observations. All data were compared with original descriptions, and specialised literature in attempt to identify all the specimens.

For the internal morphology, when necessary and feasible, the animals were dorsally/ventrally dissected and the buccal bulbs were extracted and dissolved in a solution of 10% sodium hydroxide to expose the radula. The radulae and gizzard plates were then immersed in water, dried and mounted for scanning electron microscopy (SEM) with a Nova NanoSEM 450 available at the University of Cadiz (Cadiz, Spain).

For *Runcina coronata*, in addition to the study of radula and gizzard plates, the reproductive system of specimens previously assigned to this species was examined and drawn using a dissecting microscope with the aid of a *camera lucida*. All the specimens of *R. coronata* used for morpho-anatomical study are listed in the “Examined material” sections in “Systematic description”.

RESULTS

Phylogenetic analyses

The combined dataset yielded a sequence alignment of 1475 positions (including variable sites). We obtained 189 new sequences, 64 for H3, 68 for COI and 57 for 16S (Table 1). No saturation was observed, not even in the third codon (not shown). The combined tree

(H3+COI+16S) provided better resolution than H3, COI or 16S separately (see supplementary figures S1, S2 and S3). Although bootstrap values were lower than posterior probabilities in some clades, the results of ML and BI trees rendered similar topologies.

Figure 1 shows the resulting phylogenetic hypothesis based on the combined dataset represented by Bayesian Inference and includes posterior probabilities (PP) and bootstraps support values (BS). Both ML and BI supported the monophyly of the order Runcinida (PP = 1; BS = 99), with the species *Ilbia ilbi* Burn, 1963 (family Ilbiidae) sister to Clade A (family Runcinidae). Clade A (PP = 0.96; BS = not recovered by ML) is divided into two main sister subclades, here called Clade B (PP = 0.98, not recovered by ML) and Clade C (PP = 1, BS = 78).

Clade B was subdivided into three well supported clades: the first, Clade D including only the specimen labelled as runcinid sp.2; the second, Clade E (PP = 1; BS = 96) containing *Metaruncina setoensis* (Baba, 1967) and *Metaruncina nhatrangensis* Chernyshev, 2005; and the third, Clade F (PP = 1; BS = 99), with most of *Runcina* specimens and *Pseudoilbia avellana* (Schmekel and Cappellato, 2001). Within Clade F, *Runcina ornata* appears as basal and sister to the remaining *Runcina* specimens. Within the latter, three clades with the maximum support correspond to well-established species, such as *R. lusitanica*, *R. marcosi* or the type species *R. coronata*. However, specimens belonging to *Runcina adriatica* Thompson, 1980, and *R. ferruginea* split in different well-supported clades, as well as some specimens identified as *Runcina hansbechi* Schmekel and Cappellato, 2001 and *Runcina africana* Pruvot-Fol, 1953. In addition, specimens identify as *Runcina* cf. *bahensis* Cervera, Garcia-Gomez and Garcia, 1991 and *Runcina hornae* Schmekel and Cappellato, 2002 cluster together with several unidentified species from Spain (PP = 1; BS = 100).

Clade C (PP = 1; BS = 78) was subdivided into two clades: Clade G, containing *Runcina ferruginea* Kress, 1977 with maximum support (PP = 1; BS = 100); and Clade H (PP = 1; BS = 80), including *Lapinura divae* (Ev. Marcus and Er. Marcus, 1963), *Lapinura* sp. 1, *R. ferruginea* and runcinid sp. 1 (PP = 1; BS = 82) as sister group to *Runcinida marisae* Chernyshev, 1998, *Runcinida valentinae* Chernyshev, 2006 and *Runcinida* sp. 1 (PP = 1; BS = 90). *Runcinida* sp. 1 was sister species of *Runcinida marisae* (PP = 1; BS = 100).

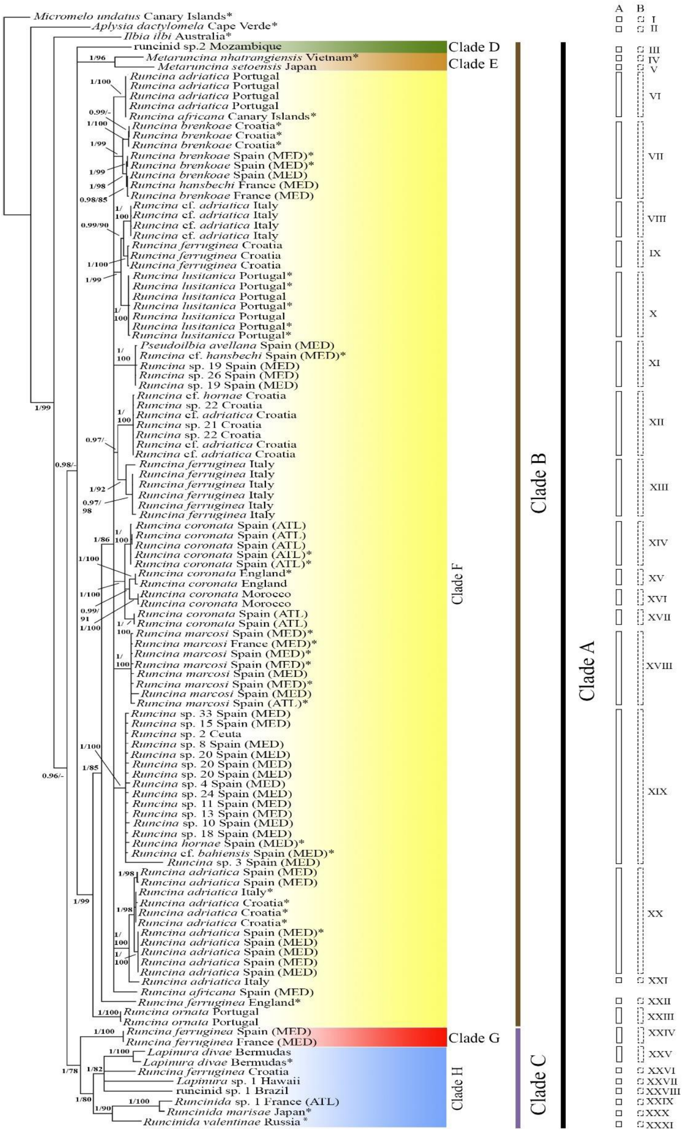

Figure 1. Phylogenetic hypothesis of the order Runcinida based on the combined dataset (H3+COI+16S) inferred by Bayesian analysis. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from Maximum likelihood analysis. (**A**) ABGD results based on the COI data set. (**B**) bPTP results based on the COI data set. Rectangles in Ilbia ilbi are missing since there is no COI sequence available. Abbreviations: ATL, Atlantic Ocean; MED, Mediterranean Sea. (*) Refers to sequences from Genbank.

Species delimitation

The ABGD analyses for COI sequences identified 31 groups in all three models of evolution (Fig. 1A). Within these groups and for all models, the specimens identified as *R. coronata* (type species of the genus *Runcina*) split into four distinct groups: RC1, RC2, RC3 and RC4 (Figure 2). The bPTP analysis showed the same result (Figs. 1B and 2).

The minimum pairwise uncorrected *p*-distances for COI among groups within Runcinida included in the present study are presented in Table 2, ranging from 12.2% to 23.1%. Within de genus *Runcina,* distances ranged from 0.0 to 17.7% (Table 2, VI–XXIII). Regarding the specimens previously identified as *R. coronata*, the distances among the above groups were considerably high compared with the distances between specimens within each group (Table 3). Groups RC1 and RC4 contain specimens from the same locality (Cádiz, Spain) and the minimum distance between them was 7.8%. Therefore, they should be considered as sympatric species. Comparing both groups with group RC2, which contains specimens from Swanage (England), the minimum distances were 6.3% for RC1 and 8.8% for RC4. Finally, the minimum genetic distances when these three groups are compared with group RC3 (specimens from Morocco) were 6.4% (RC1), 7.0% (RC2) and 9.9% (RC4) (Table 3).

Our morphological studies and molecular results showed four different species under the name *Runcina coronata.* Here, we present a redescription of *R. coronata* and the description of three new species.

Table 3. Uncorrected *p*-distances based on COI sequences from specimens previously identified as *Runcina coronata*.

	Distance between groups (%)			Distance within groups (%)
	RC1	RC2	RC3	
RC1				0.2–0.5
RC2	6.3–7.3			1.0
RC3	6.4–7.8	7.0–7.7		0.7
RC4	7.8–8.0	8.8–9.5	9.9–10.2	0.0

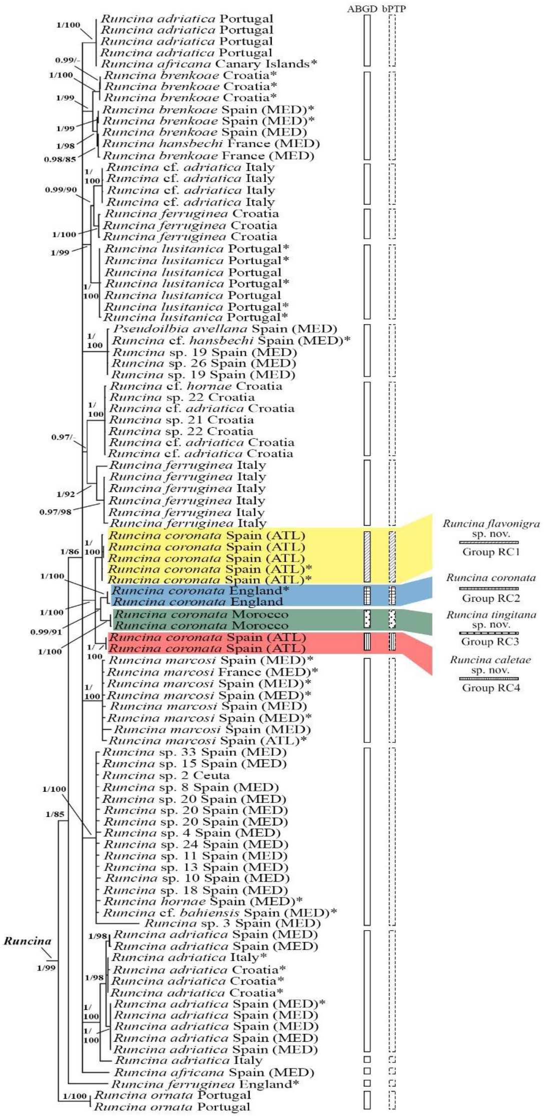

Figure 2. Detail of Clade F from analyses illustrated in Fig. 1, but focusing on species delimitation analyses of "*Runcina coronata*" species complex. Continuous rectangles, ABGD analysis based on the COI dataset. Dotted rectangles, bPTP analysis based on the COI dataset. (*) Refers to sequences from Genbank.

Systematic description

Family RUNCINIDAE H. Adams and A. Adams, 1854

Runcina Forbes in Forbes and Hanley, 1851

Runcina coronata (Quatrefages, 1844)

(Figs 3A–B; 4A–C; 5A; 6A)

Synonymy

Pelta coronata Quatrefrages, 1844, Ann. Sci. Nat. Zool. 1: 151–152, pl.3, fig. IV.

Runcina hancocki Forbes [in Forbes and Hanley], 1851, A History of British Mollusca: 611–612, pl. C.C.C, fig. 2.

R. calaritana Colosi, 1915, Mem. Reale Accad. Sci. Torino, Ser. II, 66 (6): 1–35, figs 1–18.

R. aurata García, López, Luque and Cervera, 1986, Cah. Biol. Mar. 27: 457–468, figs. 1–4.

Type locality: Bréhat, Bretagne, France.

Distribution: Bréhat, Bretagne, France (Atlantic) (Quatregafes, 1844); Torbay, England. (Alder and Hancock, 1846, Forbes, 1853); Arrábida, Portugal (Calado *et al.,* 1999); Atlantic and Mediterranean Spanish coasts (Ortea, 1976, Ballesteros and Ortea, 1981, Templado, 1984, García-Gómez *et al.*, 1986 [as *R. aurata*], Gosliner, 1990 [as *R. aurata*], Cervera, García-Gómez and García, 1991, Schmekel and Cappellato, 2002, Ballesteros *et al.*, 2016); Marseille, Mediterranean French coast (Vayssiere, 1883).

Examined material: MNCN 15.05/88105, Swanage, southern England, coll. Ian F. Smith, Apr 2016, 3 mm living animal, found brushing of the bases of stones with pink coralline algae encrusted (dissected and sequenced). MNCN 15.05/90423, Swanage, southern England, coll. Ian F. Smith, Apr 2016, 4 mm living animal, found brushing of the bases of stones with pink coralline algae encrusted (dissected and sequenced).

External morphology (Fig. 3A–B): Body elongated and moderately broad. Notum smooth. Posterior part of the notum rounded. Anterior part of the foot rounded and wide, pointed at the posterior part. Foot slightly wider than the notum. Lateral grooves on both sides between notum and foot. General ground colour of body dark brown. Front part of the notum dark brown on the central zone between the eyes and pale fawn on the sides. White chalk or yellowish spots all over the body, concentrated as semicircle bands behind the eyes and anterior to the notum end, and on the surface of the foot. Eyes small and visible. Three rounded gills laminae to the right of the anus; the largest in the middle divided into two arcs. Gills yellowish with slightly brown margins. Anus located in median line of body, beneath the end of notum.

Internal anatomy (Figs 4A–C; 5A; 6A): Radular formula 19 x 1.1.1 (MNCN 15.05/88105). Rachidian tooth bilobed with long and smooth lateral wings on each side. Central part of rachidian tooth contains a pair of pads, each one possessing 9–11 short denticles. Size and strength of the denticles vary among them. Three or four denticles developed and thick alternated with smaller and thinner ones. Central small depression presents between pads; small denticle may be present in some rows (Fig. 4A). Lateral teeth denticulate, elongate, hooked shape with 30–34 relatively long denticles (Fig. 4B). Triangular jaws present. Four gizzard plates with 10 lamellae (Fig. 4C). Shell absent. Reproductive system monoaulic. Female gland mass placed on right side and behind the digestive gland divided into two lobes. Female gland opens to exterior through median size common genital duct (Fig. 5A). Male pore opens next to mouth, on the right side. Elongated and cylindrical male copulatory organ. Penial papilla absents. Cylindrical prostate gland. Slender seminal vesicle with half size of prostate gland (Fig. 6A).

Runcina caletae **sp. nov.**

(Figs 3C–D; 4D–F; 5B; 6B)

Examined material: Holotype: MNCN (pending of number voucher), La Caleta (Cádiz), Andalusia, southwestern Spain, coll. Josep Romà, 17 May 2015, 3 mm living animal, depth 0.5–1 m (dissected and sequenced). Paratype: MNCN (pending of number voucher), La Caleta (Cádiz), Andalusia, southwestern Spain, coll. Josep Romà, 16 June 2015, 1.5 mm in length preserved, depth 0.5–1 m. (dissected and sequenced).

Etymology: The name *caletae* refers to La Caleta beach, Cádiz (Spain) where this species was found.

External morphology (Fig. 3C–D): Body elongated and broad. Anterior part of notum ("head") slightly bilobed. Posterior part of notum rounded. Foot as wide as notum. Foot extended beyond the notum on the back. Lateral grooves on both sides between notum and foot. Ground colour of body translucent pale fawn or yellowish. Digestive system visible as broad

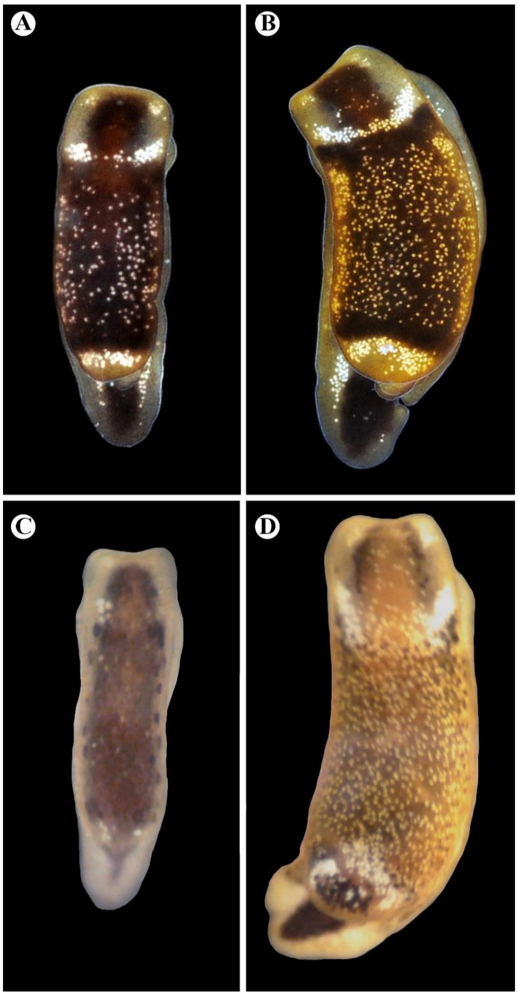

Figure 3. Living animals of *Runcina coronata* (**A, B)** and *Runcina caletae* sp. nov. (**C, D**). **A** MNCN 15.05/88105 and **B** MNCN 15.05/90423 specimens from Swanage (southern England) (photos Ian F. Smith). **C** MNCN (pending of number voucher) and **D** MNCN (pending of number voucher). Specimens from La Caleta, Cádiz (southwestern Spain; Atlantic Ocean) (photos Ana Karla Araujo).

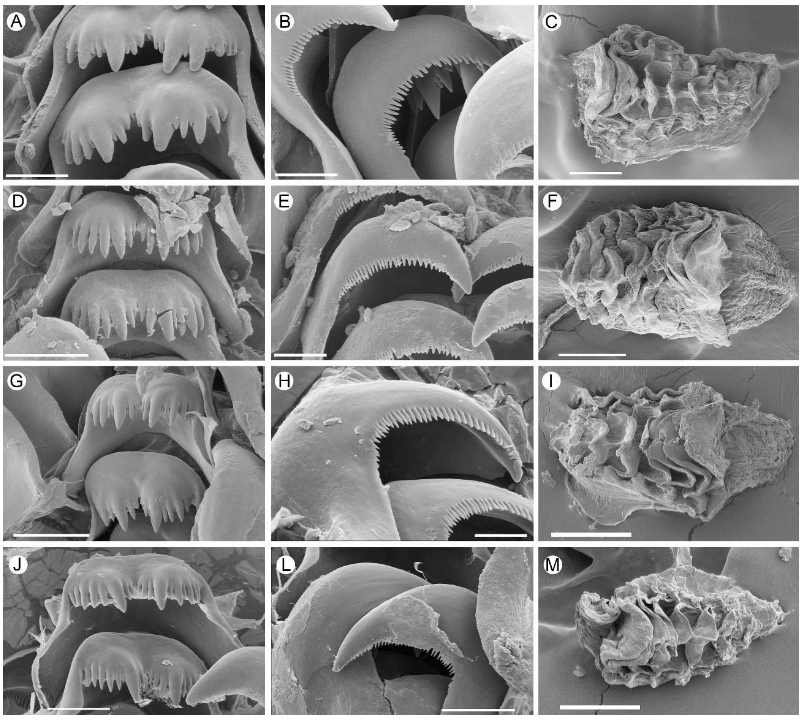

Figure 4. Scanning electron micrographs of radula and gizzard plates of "*R. coronata"* complex. **A, B, C**. *R. coronata* (MNCN 15.05/88105, MNCN 15.05/90423). **A**. Rachidian teeth (MNCN 15.05/88105). **B**. Lateral teeth (MNCN 15.05/88105). **C**. Gizzard plate (MNCN 15.05/90423). **D, E, F**. *R. caletae* sp. nov. (MNCN pending of number voucher) **D**. Rachidian teeth. **E.** Lateral teeth. **F**. Gizzard plate **G, H, I**. *R. flavonigra* sp. nov. (MNCN pending of number voucher). **G**. Rachidian teeth (MNCN pending of number voucher). **H**. Lateral teeth (MNCN pending of number voucher). **I**. Gizzard plate (MNCN pending of number voucher). **J, L, M**. *R. tingitana* sp. nov. (MNCN pending of number voucher). **J**. Rachidian teeth (MNCN pending of number voucher). **L**. Lateral teeth (MNCN pending of number voucher). **M**. Gizzard plate (MNCN pending of number voucher). Scale bars: **A, B, E, J, L** = 10 µm; **C, F, I, M** = 50 µm; **D, G** = 20 µm; **H** = 5 µm.

brownish blotch in juvenile specimen. Tiny white, black and yellow spots all over the body. White spots on the lobes of the head and on the anterior ventral surface of the foot. Triangular white patches behind the eyes. White semicircle anterior to the notum end. Very white spots few in juvenile. Small black dots forming two longitudinal lines on the head zone; concentrated on the notum end posteriorly to white semicircle. In juvenile, few big black spots on margin of notum. Eyes difficult to distinguish. Dark band on the middle of the posterior surface of the foot. Three rounded gills laminae to the right of the anus. Gills yellowish with slightly brown margins. Anus located in median line of body, beneath the end of notum.

Internal anatomy (Figs 4D–F; 5B; 6B): Radular formula 13 x 1.1.1 (MNCN pending of number voucher). Rachidian tooth slightly bilobed with long and smooth lateral wings on each side. Central part of rachidian tooth contains a pair of pads, each one possessing 7–8 denticles. Denticles long and pointed, stalactite shaped-like. Regular size, decreasing towards middle of the tooth (Fig. 4D). Lateral teeth denticulate, elongate, hooked shape with 33–36 long and pointed denticles (Fig. 4E). Triangular jaws present. Four gizzard plates with 7–10 lamellae (Fig. 4F). Shell absent. Reproductive system monoaulic. Female gland mass placed on right side and behind digestive gland. Female gland opens to exterior through common genital duct (Fig. 5B). Male pore opens next to mouth, on right side. Elongated and cylindrical male copulatory organ. Short and unarmed penial papilla projects into large atrium. Cylindrical prostate gland strongly curved. Long and slender seminal vesicle with black pigmentation (Fig. 6B).

Runcina flavonigra **sp. nov.**

(Figs 4G–I; 5C; 6C; 7A–D)

Examined material: Holotype: MNCN (pending of number voucher), La Caleta (Cádiz), Andalusia, southwestern Spain, 08 April 2019, 3.5 mm living animal, depth 0.5 – 1 m (dissected and sequenced). Paratype: MNCN 15.05/88106, La Caleta (Cádiz), Andalusia, southwestern Spain, coll. Josep Romà, 18 April 2015, 2 mm in length preserved, depth 0.5 – 1 m. (dissected and sequenced). Additional material: MNCN 15.05/88107, La Caleta (Cádiz), Andalusia southwestern, Spain, coll. Josep Romà, 18 April 2015, 2 mm in length preserved, depth 0.5 – 1 m (dissected and sequenced). MNCN (pending of number voucher), La Caleta (Cádiz), Andalusia southwestern, Spain, coll. Josep Romà, 17 May 2015, 1.5 mm in length preserved,

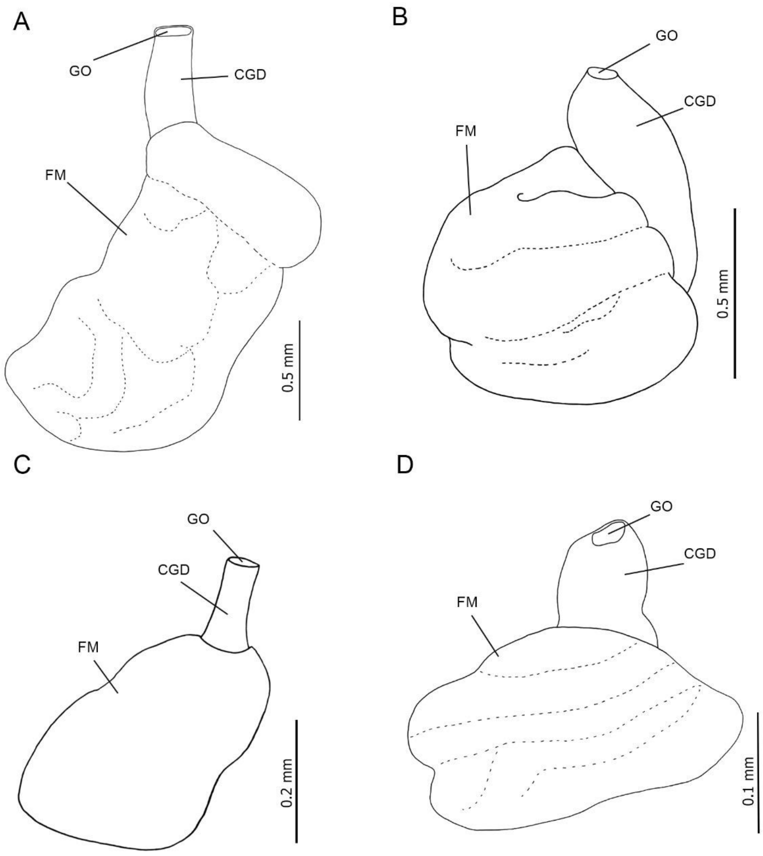

Figure 5. Female reproductive system of "*R. coronata"* complex. **A**. *R. coronata*, southern England (MNCN 15.05/90423). **B**. *R. caletae* sp. nov., La Caleta, Cádiz (southwestern Spain, Atlantic Ocean) (MNCN pending of number voucher). **C**. *R. flavonigra* sp. nov., La Caleta, Cádiz (southwestern Spain, Atlantic Ocean) (MNCN pending of number voucher). **D.** *R. tingitana* sp. nov., Tangier (northwestern Morocco, Atlantic Ocean) (MNCN pending of number voucher). Abbreviations: FM, female mass; CGD, common genital duct; GO, gonopore.

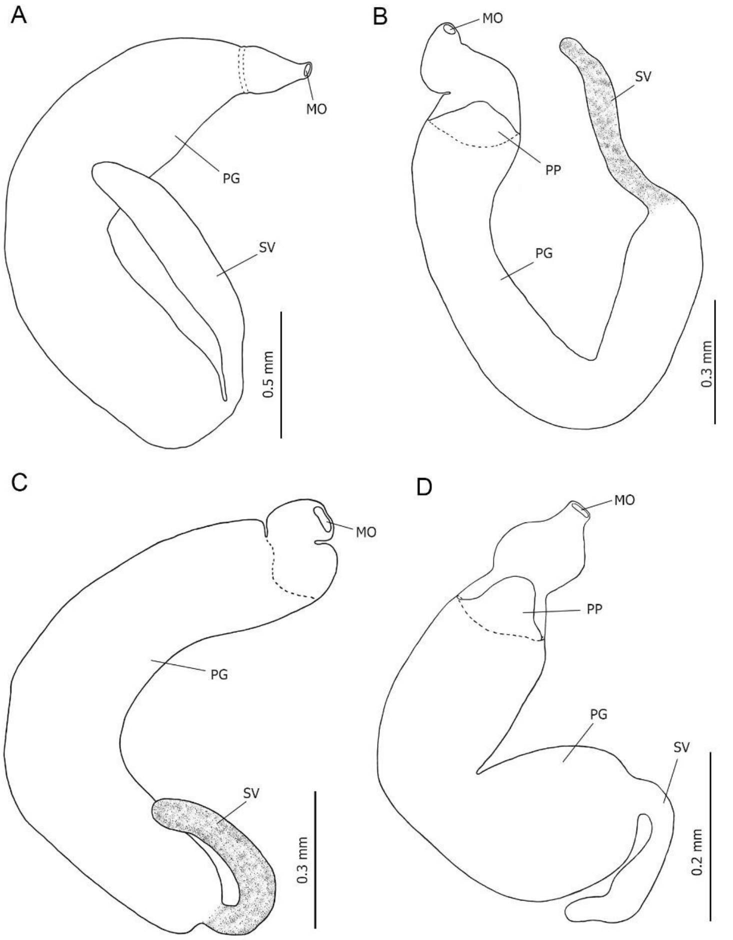

Figure 6. Male reproductive system of "*R. coronata*" complex. **A**. *R. coronata*, southern England (MNCN 15.05/90423). **B**. *R. caletae* sp. nov., La Caleta, Cádiz (southwestern Spain, Atlantic Ocean) (MNCN pending of number voucher). **C**. *R. flavonigra* sp. nov., La Caleta, Cádiz (southwestern Spain, Atlantic Ocean) (MNCN pending of number voucher). **D.** *R. tingitana* sp. nov., Tangier (northwestern Morocco, Atlantic Ocean) (MNCN pending of number voucher). Abbreviations: MO, male opening; PP, penial papilla; PG, prostate gland; SV, seminal vesicle.

depth 0.5 – 1 m (dissected and sequenced). MNCN (pending of number voucher), El Chato (Cádiz), Andalusia southwestern, Spain, coll. Ana Bartual, 13 April 2015, 1 mm in length preserved, depth 0.5 – 1 m (dissected and sequenced).

Etymology: The specific name *flavonigra* refers to its yellow colour and the black spots on the notum (from Latin: *flavus*, yellow; *nigrum*, black).

External morphology (Fig. 7A–D): Body elongated and moderately broad. Anterior part of notum ("head") slightly bilobed. Posterior part of notum rounded. Anterior part of foot rounded and pointed at posterior part. Foot as wide as notum. Foot extended beyond notum on the back. Lateral grooves on both sides between notum and foot. Ground colour of body translucent pale fawn or yellowish. Digestive system visible as a broad brownish blotch. White spots on central zone of notum, behind eyes forming triangular patches and anterior to notum end. White spots maybe also absent. Black dots dispersed on notum and more concentrated on head zone. Eyes difficult to distinguish. Dark band on middle of dorsal surface of foot. Black dots may be present on ventral surface of foot. Four rounded and relatively large gills laminae to the right of anus. Gills yellowish with slightly brown margins. Anus located in median line of body, beneath the end of notum.

Internal anatomy (Figs 4G–I; 5C; 6C): Radular formulae 12 x 1.1.1 (MNCN pending of number voucher) and 13 x 1.1.1 (MNCN pending of number voucher). Rachidian tooth bilobed with long and smooth lateral wings on each side. Central part contains pair of pads, each one possessing 10–11 long, slender, pointed denticles. Size and strength of denticles vary among them. Large and small denticles arranged alternately. Central small depression present between pads; small denticle seems to be present (Fig. 4G). Lateral teeth denticulate, elongate, hooked shape with 35–36 long and pointed denticles (Fig. 4H). Triangular jaws present. Four gizzard plates with 7–9 lamellae (Fig. 4I). Shell absent. Reproductive system monoaulic. Female gland mass placed on right side and behind digestive gland, opening to exterior through small size common genital duct (Fig. 5C). Male pore opens next to mouth, on the right side. Elongated and cylindrical male copulatory organ. Penial papilla absents. Cylindrical and long prostate gland ends in slender and small seminal vesicle with black pigmentation (Fig. 6C).

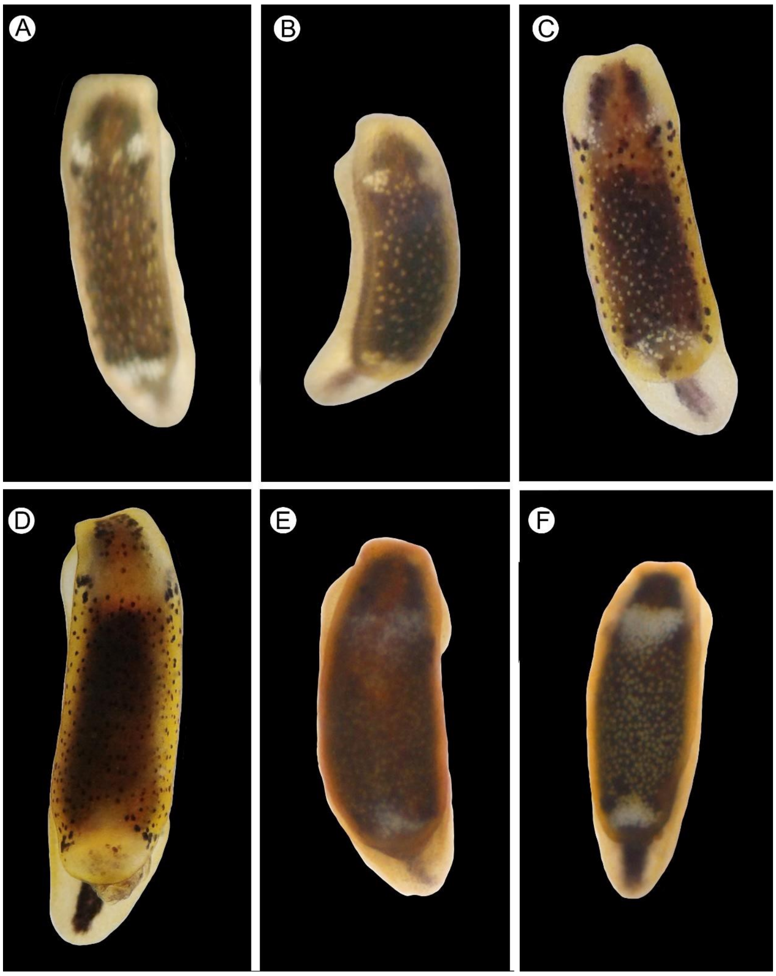

Figure 7. Living animals of *Runcina flavonigra* sp. nov. (**A–D)** and *Runcina tingitana* sp. nov. (**E, F**). **A** (MNCN pending of number voucher), **B** (MNCN pending of number voucher), **C** (MNCN pending of number voucher) and **D** (MNCN pending of number voucher): specimens from La Caleta, Cádiz (southwestern Spain; Atlantic Ocean) (photos Ana Karla Araujo). **E** (MNCN pending of number voucher) and **F** (MNCN pending of number voucher): specimens from Tangier (northwestern Morocco; Atlantic Ocean) (photos Naoufal Tamsouri).

Runcina tingitana sp. nov.

(Figs 4J–M; 5D; 6D; 7E–F)

Examined material: Holotype: MNCN (pending of number voucher), Tangier, Morocco, coll. Naoufal Tamsouri, 22 March 2018, 2 mm living animal, depth 0.5 – 1 m (dissected and sequenced). Paratype: MNCN (pending of number voucher), Tangier, Morocco, coll. Naoufal Tamsouri, 22 March 2018, 1.5 mm fixed animal, depth 0.5 – 1 m (dissected and sequenced).
Etymology: The name *tingitana* refers to Tingi, the Roman name of Tangier (Morocco), the type locality of the species.

External morphology (Fig. 7E–F): Body elongated. Anterior part of notum ("head") straight, rounded on posterior part. Foot as wide as notum. Anterior and posterior parts rounded. Foot extended beyond notum posteriorly. Lateral grooves on both sides between notum and foot. Ground colour of the body light brown. Digestive system visible as a broad brownish blotch. White spots concentrated behind eyes and anterior to notum end forming a triangle. Yellowish dots dispersed on middle of notum. Some dark small spots can be present posterior to white spots at head and anterior to white spots at notum end. Eyes difficult to distinguish. Dark band on middle of dorsal surface of foot. Two rounded gills laminae on right side of anus. Gills light brown. Anus located in median line of body, beneath the end of notum.

Internal anatomy (Figs 4J–M; 5D; 6D): Radular formulae 14 x 1.1.1 (MNCN pending of number voucher) and 12 x 1.1.1 (MNCN pending of number voucher). Rachidian tooth bilobed with smooth lateral wings on each side. Central part of rachidian tooth contains pair of pads, each one possessing 7–10 denticles. Denticles short and triangular. The third or fourth denticle more developed. Central small depression present between pads; small denticle absent (Fig. 4J). Lateral teeth denticulate, elongate and hooked shaped with 34–37 long, thin, pointed denticles (Fig 4L.). Triangular jaws present. Four gizzard plates with 8 lamellae (Fig. 4M). Shell absent. Reproductive system monaulic. Female gland mass placed on right side and behind digestive gland. Opens to exterior through short and wide common genital duct (Fig. 5D). Male pore opens next to mouth, on right side. Male copulatory organ cylindrical. Short and unarmed penial papilla projects into round atrium. Prostate gland cylindrical and strongly curved with posterior part rounded. Seminal vesicle slender with middle part wider (Fig. 6D).

DISCUSSION

Based on morpho-anatomical characters, in particular shell, gills and radula, Burn (1963) presented a systematic classification for the order Runcinida. According to his classification, the rare *Ildica nana* Bergh, 1889 was the most primitive species followed by the genera *Runcina*, *Runcinida* and *Runcinella*, while the genus *Ilbia* was considered by Burn as the recent most. This classification diverges in part from our results, based on molecular phylogeny, where *Ilbia* appears to be more primitive than the genera *Runcina* and *Runcinida*. Unfortunately, since no material of *Ildica* was available, we cannot agree or disagree with Burn's proposal that this genus is the most primitive among Runcinida genera. In our results all members of Runcinida cluster together in highly supported clade (PP = 1; BS = 99). Concerning the families Ilbiidae and Runcinidae it is necessary more taxa of both families to better understand and discuss their state. Although there is some agreement on the use of characters such as the radula, gills and shell to identify species and genera, in some cases they appear not to be accurate or enough. Due to their small size and dark, dull cryptic colour pattern in most species, to detect and identify runcinids can be very challenging and, also, lead to erroneous identifications.

***Runcina divae* or *Lapinura divae*?**

The genus *Lapinura* introduced by Er. Marcus and Ev. Marcus (1970) is specially characterized by an external cup-shaped larval shell. These genus was synonymised with *Runcina* by Clark (1984) after examining populations of supposed *Lapinura divae* in which some specimens lacked the external shell. Because of this "variability", Clark disregarded the external shell and others characteristics that define the genus, emphasising only the radular formula (N x 1.1.1) and the presence of gizzard plates – common for most of runcinids – to place *Lapinura divae* in *Runcina*.

Although our study has as its main focus the European runcinids, we observe that *Lapinura divae* clusters out of Clade F (most of *Runcina* specimens) and joins other runcinids in a clade we called Clade H. As shown in Figure 1, *Lapinura* and *Runcina* are not close related; therefore, we conclude that *Lapinura divae* should be used for the Caribbean species described by Ev. Marcus and Er. Marcus (1963). Moreover, we agree with Ortea *et al*. (2017) about a possible existence of more than one species under the name *Lapinura divae*, due to the discrepancies among specimens collected in the Caribbean Sea and Brazil (Ev. Marcus and Er. Marcus, 1963, Er. Marcus and Ev. Marcus, 1970, Thompson, 1977, Clark, 1984).

What about European runcinids?

Three genera are present in European waters: *Runcina* (31 species)*, Runcinella* (one species) and *Pseudoilbia* (one species). We have analysed specimens of *Runcina* and *Pseudoilbia*, but unfortunately some species of *Runcina* and specimens of *Runcinella condio* Moro & Ortea, 2015 described from Canary Island were not available (see Taxon Sampling section).

To date, the genus *Runcinida* was restricted to western Pacific Ocean and included three species, *R. elioti* (Baba, 1937), *R. valentinae* and *R. marisae*. However, according to our results, the genus *Runcinida* is also present in European waters, since the specimen identified as *Runcinida* sp. 1, collected in Cap Ferret, France (Atlantic coast), clustered together with *R. marisae* and *R. valentinae*. The type species of *Runcinida* was originally described as *Runcina elioti*, but Burn (1963) removed this species from *Runcina* and erected the new genus *Runcinida*, based on the shape, arrangement and position of the gill. Externally, all species of *Runcinida* bear the same main body colour, dark-brown notum and yellowish on the margin and foot, also with gills arranged in semicircle above the anus. Our specimen (*Runcinida* sp. 1) fits this colour pattern and gills arrangement, but differs from the others three described *Runcinida* by having small black dots, especially on the dorsal and ventral surfaces of the foot and several white spots on the notum. *Runcinida elioti* (Baba, 1937) has fewer white spots, while small black dots on the foot are missing (Baba, 1937). *Runcinida marisae* has small black dots on the ventral surface of the foot and white dots are absent (Chernyshev, 1998). Finally, in *Runcinida valentinae* both the small black spots and white dots are missing; however, it presents a triangular orange patch on the anterior part of the notum (Chernyshev, 2006). The ABDG and bPTP analyses split *Runcinida* sp. 1, *R. marisae* and *R. valentinae* in three different groups (Fig. 1, groups XXIX–XXXI). Moreover, the uncorrected *p*-distances among the three species range from 6.5% to 16.6% (Table 2). Considering the external differences, the geographical distribution and the results of our phylogenetic analyses, we conclude that our specimen from the Atlantic coast of France is a distinct undescribed species of *Runcinida*, close related to *R. marisae.*

Pseudoilbia

The species *Pseudoilbia avellana*, the only representative of this genus in Europe, was originally described as *Runcina avellana* by Schmekel and Cappellato (2001). These authors found an unusual radula (3 x 1.0.1) in the single specimen examined which they pointed as an alleged juvenile. Besides, no gizzard plates were found. Based on this information, Ortea (2013) suggested that *R. avellana* should be transferred to the genus *Pseudoilbia* due to the absence of rachidian tooth and gizzard plates, and proposed the new combination *Pseudoilbia avellana.* According to our phylogenetic analyses, the species *Pseudoilbia avellana* clusters together with *Runcina* species, which means that the combination proposed by Ortea (2013) is not correct. Thus, we reinstate the name *Runcina avellana* for this species. However, considering that Schmekel and Cappellato (2001) examined only one specimen, they could have observed an aberrant radula, which has occurred in others heterobranchs as *Glaucus atlanticus* Foster, 1777 (Valdés and Angulo-Campillo, 2004) or *Nembrotha chamberlaini* Gosliner and Behrens, 1997 (Pola *et al.*, 2008), for example. More specimens of *Runcina avellana* and a detailed examination of their internal anatomy are certainly required in order to clarify the information about radula and gizzard plates of this species.

Runcina

The genus *Runcina* is characterized by up to four sperated gills on the right side of the anus and triseriate (1.1.1) radula with bilobed rachidian teeth and smooth or denticulated lateral teeth. According to our analysis, the monophyly of *Runcina* is supported (PP = 1; BS = 99) only when *R. ferruginea* (northeastern, Spain), *R. ferruginea* (France, Mediterranean) and *R. ferruginea* (Croatia) are excluded. These three specimens cluster with others runcinids in the Clade C. They were clearly misidentified as *Runcina* spp.

In our analysis, we have included specimens early identified as *Runcina adriatica, R. africana, R.* cf. *bahiensis, R. brenkoae* Thompson, 1980*, R. coronata, R. ferruginea, R.* cf. *hansbechi* Schmekel and Cappellato, 2001*, R. hornae, R. lusitanica* Araujo, Pola, Malaquias and Cervera, 2019 (see Chapter II), *R. marcosi* Araujo, Pola, Malaquias and Cervera, 2019 (see Chapter II), *R. ornata*, *R. avellana* (see discussion above) which represents 38,7% of nominal species of European *Runcina*. In addition, several unidentified specimens of *Runcina* from Spain, Italy and Croatia were also included in the analysis. As stated previously, species from Canary Islands were not available and also represent 38,7% of European species of *Runcina*.

Within *Runcina*, we obtained one clade with maximum support (PP = 1; BS = 100) that gathers together one specimen of *Runcina* cf. *bahiensis*, one specimen of *Runcina hornae* and several unidentified specimens. Most specimens in this clade were collected in Catalonia (northeastern Mediterranean Spanish coast) and despite their remarkable variation on the colour pattern (Fig. 8), our species delimitation analysis show that they belong to the same species (uncorrected p-distances = 0.0–2.0% between specimens). *Runcina bahiensis* was originally described from Bay of Algeciras (Strait of Gibraltar, Spain) (Cervera *et al.*, 1991) and *R. hornae* from Banyuls-sur-Mer (Mediterranean coast of France) (Schmekel and Cappellato, 2002). Both species have been reported in several localities in Catalonia (Sánchez-Moyano *et al*., 2000, Ballesteros *et al*., 2016). *R. bahiensis* differs from *R. hornae* by the colour pattern, number of rows of radular teeth, shape of the body and presence of small protuberances. Some of our specimens (Fig. 8B, C and D) bear a colour pattern and body shape similar to *R. bahiensis*, but none of them present the small protuberances characteristic for this species. We examined the radula of one specimen (*Runcina* sp. 3) from Catalonia and it matches the original description of the radula of *R. hornae* (Fig. 9).

Unfortunately, specimens of *R. bahiensis* from the type locality or surroundings were not available for this study. Considering the radula and the proximity to the type locality, we assume that the specimens of this clade belong to *R. hornae* and, therefore, this species shows great colour pattern variability.

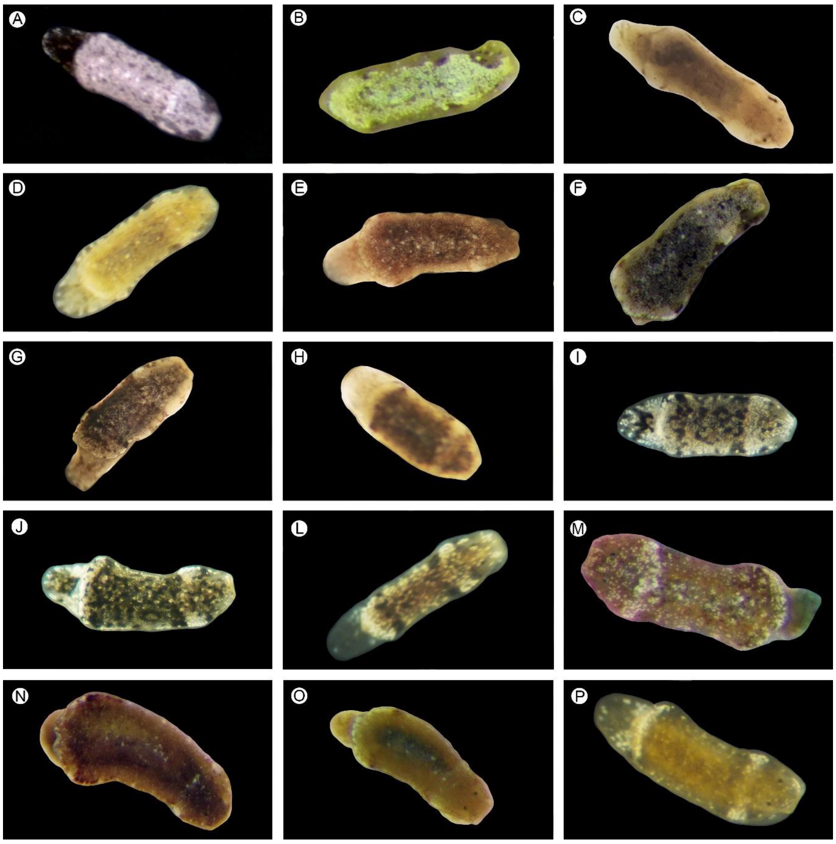

Figure 8. Living animals of *Runcina hornae*. Specimens from Catalonia, northeastern Spain (Mediterranean Sea) (photos Ana Karla Araujo [C, D, I, J, L, P], Carlés Galià [A, E, G, H], Marina Poddubetskaia [B, F, M, N, O]). **A** MNCN 15.05/88104. **B** MNCN 15.05/90661. **C** MNCN (pending of number voucher). **D** MNCN 15.05/90656. **E** MNCN 15.05/90660. **F** MNCN 15.05/90655. **G** MNCN 15.05/90659. **H** MNCN 15.05/90665. **I** MNCN 15.05/90658. **J** MNCN 15.05/90654. **L** MNCN 15.05/90657. **M** MNCN 15.05/90662. **N** MNCN 15.05/90664. **O** MNCN 15.05/90663. **P** MNCN 15.05/88110.

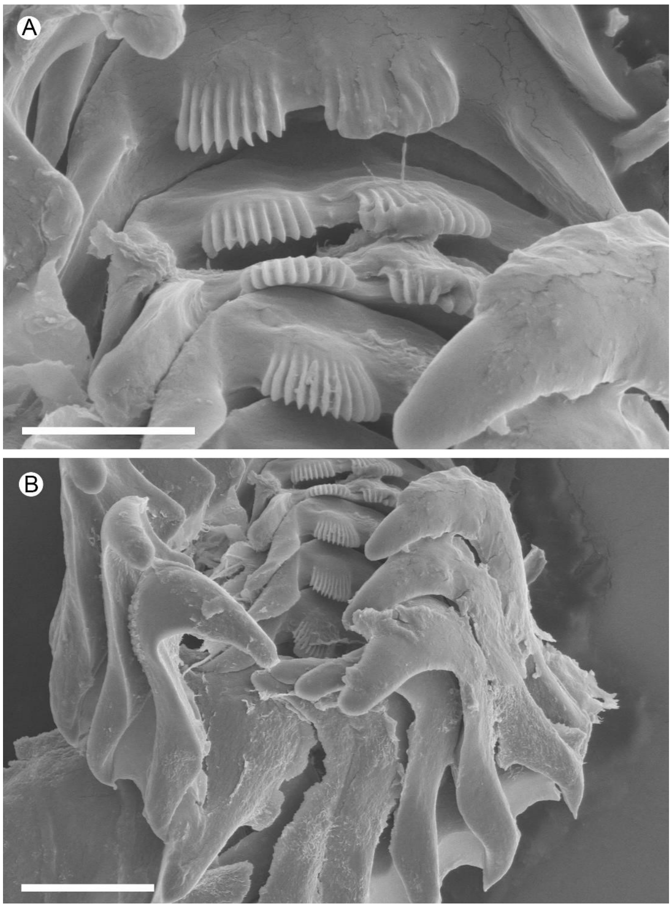

Figure 9. Scanning electron micrographs of radula of *Runcina hornae*. **A**. Rachidian teeth (MNCN 15.05/90654). **B**. Lateral teeth (MNCN 15.05/90654). Scale bars: **A** = 5 µm; **B** = 10 µm.

In the last few years, several complexes of species have been detected among heterobranch sea slugs (see among others Jörger and Schrödl, 2013, Padula *et al.*, 2014, Carmona *et al.*, 2015, Krug *et al.*, 2016, Korshunova *et al.*, 2017, Austin *et al.*, 2018). In Runcinida, the first complex of species was detected by Araujo *et al*. (2019) (see Chapter II) regarding to the species *Runcina brenkoae*, with the description of two new species, *Runcina marcosi* and *Runcina lusitanica*. In the current study, our results revealed that *R. coronata* (see

Discussion below), *R. ferruginea* (see Chapter III) and *R. adriatica* (see Chapter IV) are also part of this complex hidden diversity.

Runcina coronata species complex

In this chapter, we focused on the *Runcina coronata* complex due to the remarkable confusion surrounding the type species of the genus. Our study recognized four distinct species, namely the proper *R. coronata* (Quatrefrages, 1844), and three new species here described as *R. caletae* sp. nov., *R. flavonigra* sp. nov. and *R. tingitana* sp. nov.

Externally, all the species within this complex are quite similar in colour pattern. *R. caletae* sp. nov. and *R. flavonigra* sp. nov., are almost impossible to distinguish (Figs 3 and 7; Table 4), but differ from the other species of the complex by having translucent yellowish colour and black spots all over the notum and sometimes on the ventral surface of the foot. The colour pattern of these two species resembles that described for *Runcina aurata* Garcia, Lopez, Luque and Cervera, 1996 [synonymized with *R. coronata* by Cervera *et al*., 1991] and also the pattern found in the specimens of *R. coronata* from the Mediterranean Sea described by Vayssière (1883); however, the latter presents dark colour on the lateral sides of the notum (Vayssière, 1883, pl. 1, fig. 1). Burn (1963) suggested that the Mediterranean specimens assigned to *R. coronata* could belong to a different species due to the colour pattern, shape of the notum and number of lamellae of the gizzard plates.

Schmekel and Cappellato (2002) published a description of *R. coronata*, including scanning electron microscopy of the radula and gizzard plates, using specimens from nearby the type locality (Bretagne, France, Atlantic Ocean) and also from Plymouth (southern England, Atlantic Ocean). Our specimens from England are very similar to those described by Schmekel and Cappellato, which are characterized by lacking the black spots on the notum, especially on the head zone where the eyes are totally distinguishable, contrary to the other three species described here (Figs. 3 and 7) and *R. coronata* from Azores Island (Portugal) and Andalusia (southern Spain) that bear black spots on the notum (Garcia *et al*, 1986, Gosliner, 1990, Cervera *et al.*, 1991). In *R. tingitana* sp. nov, the black spots are difficult to distinguish due to its brownish ground colour (Fig 7). All four species within this species complex also show some differences respect to the gills (Table 4).

The internal characters may not provide substantial information to clearly distinguish the species of the "*R. coronata*" complex, but still show subtle differences. The rachidian teeth in *R. caletae* sp. nov. lack the depression between the pads and the masticatory edge show denticles with the same size, unlike the other three species (Fig. 4A, D, G, J; Table 4). *R.*

coronata, *R. flavonigra* sp. nov. and *R. tingitana* sp. nov. have rachidian teeth very similar to those described for *R. coronata* by Garcia *et al*. (1986), Cervera *et al.* (1991) and Schmekel and Cappellato (2002). However, these three species differ from each other by the size and thickness of the most developed denticles of the masticatory edge and by the presence or absence of a small denticle at the depression between the pads (Fig. 4A, G, J; Table 4). All four species differ in the number of crests on the gizzard plates (Fig. 4C, F, I, M; Table 4).

In runcinids, the male copulatory organ is separated from the rest of the reproductive system and opens at the right side of the mouth. Usually, consist in three parts: atrium, prostate gland and seminal vesicle (Ghiselin, 1963, Er. Marcus and Ev. Marcus, 1970, Kress, 1977). The original description of *R. coronata*, Quatrefages (1844) mentions this organ as “a rather short testicular bag in the shape of a club” and that the seminal vesicle may be absent. Alder and Hancock (1846) did not mentioned any detail about this organ, neither Cervera et al. (1991) or Schmekel and Cappellato (2002). In our specimens of *R. coronata*, the male copulatory organ is relatively large (2 mm in a 4 mm living animal) and the seminal vesicle is present. In general, the female reproductive system of runcinids is poorly studied and only in a few species as *Lapinura divae*, *Metaruncina setoensis* and *Runcina ferruginea* is well detailed. Here, in all four species of the “*R. coronata”* complex, the female part consists of a female mass, which opens outside to a common genital duct (Fig. 5). Some runcinids present bursa copulatrix, for example *R. australis* and *R. macfarlandi* (Burn, 1963, Gosliner, 1991). Vayssière (1883) described the ampulla and bursa copulatrix for *R. coronata*, which in our specimens could not be found. In the original description of *R. coronata*, the female part is not detailed. In the species of the “*R. coronata*” complex, the differences in the reproductive system are mainly related to the shape and size of the organs (Figs 4 and 5). However, it is necessary to highlight that the fixation method could influence these differences.

In conclusion, this study revealed a significant hidden biodiversity among runcinids in European waters. Several complexes of cryptic/pseudocryptic species were detected, including the type species of the genus *Runcina*, the largest in number of species in Europe. The discovery of this hidden biodiversity reflects that there is still so much to be discovered and, consequently, the importance of the taxonomic studies, since this type of knowledge is critical for many scientific areas as ecology, conservation, medicine, pharmacology, among others. In addition, this study provides the first overview about the relationships within the order Runcinida and highlights the necessity of further phylogenetic analyses with a wider taxon sampling for a better understanding.

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Table 1. List of specimens used for phylogenetic analysis. (*) New sequences generated for this study. Museum abbreviations: Museum Victoria collections (NMVF), University Museum of Bergen (ZMBN), Zoologische Staatssammlung München, Germany (ZSM), Museo Nacional de Ciencias Naturales (MNCN) and The Natural History Museum, London, United Kingdom (NHMUK).

Species Preliminary Ids	ID personal	Locality	Voucher no.	16S	COI	H3
Ilbia ilbi Burn, 1963		Hamers Haven, Australia	NMVF234189	MK323019	-	MK322992
Lapinura divae (Ev. Marcus & Er. Marcus, 1963)		Bermuda (GB)	ZMBN 82997	KJ022825	KF992195	KJ022893
Lapinura divae		Bermuda (GB)	ZMBN 82997	*	-	*
Metaruncina nhatrangiensis Chernyshev, 2005		Vietman (GB)	-	KP313664	KP313668	-
Metaruncina setoensis (Baba, 1954)		Okinawa, Japan	ZMBN 88231	*	*	-
Pseudoilbia avellana (Schmekel and Cappellato, 2001)	*R. avellana*	Palamos, Spain	MNCN 15.05/90415	-	*	*
Runcina adriatica T. Thompson, 1980		Azores Islands, Portugal	Pending number	-	*	*
Runcina adriatica		Azores Islands, Portugal	ZMBN81756	-	*	*
Runcina adriatica		Azores Islands, Portugal	ZMBN81756	*	*	*
Runcina adriatica		Azores Islands, Portugal	Pending number	*	-	*
Runcina adriatica		Iz Island, Croatia	MNCN 15.05/88099	MK323015	MK322986	MK323036
Runcina adriatica		Ugljan Island, Croatia	MNCN 15.05/88100	MK322996	MK322967	MK323022
Runcina adriatica		Zut Island, Croatia	MNCN 15.05/88101	MK322997	MK322968	-
Runcina adriatica		Otranto, Italy	MNCN 15.05/88102	MK322993	MK322966	-

Table 1. Continued.

Species Preliminary Ids	Revised Ids	Locality	Voucher no.	16S	COI	H3
Runcina adriatica		Palamós, Spain	MNCN 15.05/90416	-	*	*
Runcina adriatica		Cadaqués, Spain	MNCN 15.05/88103	MK322994	MK322987	MK323020
Runcina adriatica		Cadaqués, Spain	MNCN 15.05/90417	*	*	*
Runcina adriatica		Cadaqués, Spain	MNCN 15.05/90418	*	*	*
Runcina adriatica		Otranto, Italy	MNCN 15.05/90666	*	*	*
Runcina cf. *adriatica*		Lecce, Italy	Pending number	*	*	*
Runcina cf. *adriatica*		Lecce, Italy	Pending number	*	*	*
Runcina cf. *adriatica*		Lecce, Italy	Pending number	*	*	*
Runcina cf. *adriatica*		La Strea, Italy	Pending number	*	*	*
Runcina brenkoae T. Thompson, 1980		Split, Croatia	MNCN 15.05/88086	MK322998	MK322969	MK323023
Runcina africana Pruvot-Fol, 1953		Tenerife, Canary Islands, Spain	NHMUK 20030791/1	KJ022780	DQ974680	KJ022942
Runcina africana		Port Lligant, Spain	MNCN 15.05/90651	*	-	*
Runcina cf. *bahiensis* Cervera, Garcia-Gomez and Garcia, 1991	*R. hornae*	Cadaqués, Spain	MNCN 15.05/88104		MK322988	MK323005
Runcina brenkoae T. Thompson, 1980		Split, Croatia	MNCN 15.05/88086	MK322998	MK322969	MK323023
Runcina brenkoae		Nin, Croatia	MNCN 15.05/88089	MK323017	MK322972	MK323038
Runcina brenkoae		Nin, Croatia	MNCN 15.05/88088	MK323018	MK322971	MK323039

Table 1. Continued.

Species Preliminary Ids	Revised Ids	Locality	Voucher no.	16S	COI	H3
Runcina brenkoae		Roses, Spain	MNCN 15.05/88090	MK323003	MK322964	-
Runcina brenkoae		Roses, Spain	MNCN 15.05/90422	-	*	*
Runcina brenkoae		Banyuls-sur-Mer	ZSMMOL20142072	*	*	-
Runcina coronata (Quatrefages, 1844)		Swanage, England	MNCN 15.05/88105	MK323031	MK322976	MK323010
Runcina coronata		Swanage, England	MNCN 15.05/90423	-	*	*
Runcina coronata	*R. flavonigra* sp. nov	Cádiz, Spain	MNCN 15.05/88106	MN057638	-	MN057641
Runcina coronata	*R. flavonigra* sp. nov	Cádiz, Spain	MNCN 15.05/88107	MN057639	MN057640	MN057642
Runcina coronata	*R. flavonigra* sp. nov	Cádiz, Spain	Pending number	*	*	*
Runcina coronata	*R. flavonigra* sp. nov	Cádiz, Spain	Pending number	*	*	*
Runcina coronata	*R. flavonigra* sp. nov	Cádiz, Spain	Pending number	*	*	*
Runcina coronata	*R. caletae* sp. nov	Cádiz, Spain	Pending number	*	*	*
Runcina coronata	*R. caletae* sp. nov	Cádiz, Spain	Pending number	*	*	*
Runcina coronata	*R. tingitana* sp. nov	Tangier, Morocco	Pending number	*	*	*
Runcina coronata	*R. tingitana* sp. nov	Tangier, Morocco	Pending number	*	*	*
Runcina ferruginea Kress, 1977		Newlyn, Cornwall, England	MNCN 15.05/88109	MK323032	MK322977	MK323011
Runcina ferruginea		Roses, Spain	Pending number	*	*	-
Runcina ferruginea		Split, Croatia	Pending number	*	*	-

Table 1. Continued.

Species Preliminary Ids	Revised Ids	Locality	Voucher no.	16S	COI	H3
Runcina ferruginea		Lecce, Italy	Pending number	-	*	*
Runcina ferruginea		Lecce, Italy	Pending number	-	*	-
Runcina ferruginea		Lecce, Italy	Pending number	-	*	*
Runcina ferruginea		Lecce, Italy	Pending number	-	*	-
Runcina ferruginea		Lecce, Italy	Pending number	-	*	-
Runcina ferruginea		Lecce, Italy	Pending number	*	-	*
Runcina ferruginea		Iz Island, Croatia	MNCN 15.05/90667	*	*	*
Runcina ferruginea		Ugljan Island, Croatia	MNCN 15.05/90668	*	*	*
Runcina ferruginea		Split, Croatia	MNCN 15.05/90669	-	*	*
Runcina hansbechi Schmekel and Cappellato, 2001	*R. brenkoae*	Banyuls-sur-Mer, France	ZSMMOL20142071	*	*	*
Runcina cf. *hansbechi*	*R. avellana*	Mataró, Spain	MNCN 15.05/88108	MK323028	MK322973	MK32300
Runcina hornae Schmeckel and Cappellato, 2002		Palamós, Spain	MNCN 15.05/88110	MK323033	MK322978	-
Runcina lusitanica Araujo, Pola, Malaquias and Cervera, 2019		Faro, Portugal	MNCN 15.05/88091	MK323024	MK322979	MK322999
Runcina lusitanica		Faro, Portugal	MNCN 15.05/200065	-	MK322981	MK323002
Runcina lusitanica		Faro, Portugal	MNCN 15.05/88093	-	MK322965	MK323004

Table 1. Continued.

Species Preliminary Ids	Revised Ids	Locality	Voucher no.	16S	COI	H3
Runcina lusitanica		Faro, Portugal	MNCN 15.05/88092	MK323035	MK322980	MK323014
Runcina lusitanica		Faro, Portugal	MNCN 15.05/88094	MK323027	MK322989	-
Runcina lusitanica		Faro, Portugal	MNCN 15.05/90424	*	*	*
Runcina lusitanica		Faro, Portugal	MNCN 15.05/90425	*	*	*
Runcina marcosi Araujo, Pola, Malaquias and Cervera, 2019		Roses, Spain	MNCN 15.05/88098	MK323025	MK322982	MK323000
Runcina marcosi		Mataro, Spain	MNCN 15.05/88095	MK323037	MK322983	MK323016
Runcina marcosi		Roses, Spain	MNCN 15.05/88097	-	MK322990	MK323012
Runcina marcosi		Roses, Spain	MNCN 15.05/88096	MK323034	MK322984	MK323013
Runcina marcosi		Cadiz, Spain	MNCN 15.05/200066	-	MK322985	MK323006
Runcina marcosi		Banyuls-sur-Mer, France	ZSM MOL 201442089	MK323021	MK322991	MK322995
Runcina marcosi		Roses, Spain	MNCN 15.05/90426	-	*	-
Runcina marcosi		Roses, Spain	MNCN 15.05/90427	*	*	*
Runcina ornata (Quatrefages, 1844)		Azores Islands, Portugal	ZMBN87929	*	*	-
Runcina ornata		Azores Islands, Portugal	ZMBN87949	*	*	-
Runcina sp. 2	*R. hornae*	Ceuta, Spain	Pending number	*	-	*
Runcina sp. 3	*R. hornae*	Mataro, Spain	MNCN 15.05/90654	*	*	*
Runcina sp. 4	*R. hornae*	Palamos, Spain	Pending number	-	*	*

Table 1. Continued.

Species Preliminary Ids	Revised Ids	Locality	Voucher no.	16S	COI	H3
Runcina sp. 8	*R. hornae*	Roses, Spain	MNCN 15.05/90655	*	*	*
Runcina sp. 10	*R. hornae*	Mataro, Spain	MNCN 15.05/90656	*	*	*
Runcina sp. 11	*R. hornae*	Mataro, Spain	MNCN 15.05/90657	*	*	*
Runcina sp. 13	*R. hornae*	Mataro, Spain	MNCN 15.05/90658	-	*	*
Runcina sp. 15	*R. hornae*	Cadaqués, Spain	MNCN 15.05/90659	-	*	*
Runcina sp. 17	*R. adriatica*	Cadaqués, Spain	MNCN 15.05/90419	*	*	*
Runcina sp. 18	*R. hornae*	Cadaqués, Spain	MNCN 15.05/90660	*	*	*
Runcina sp. 19	*R. avellana*	Roses, Spain	Pending number	*	*	-
Runcina sp. 19	*R. avellana*	Roses, Spain	MNCN 15.05/90652	*	*	*
Runcina sp. 20	*R. hornae*	Roses, Spain	MNCN 15.05/90661	*	*	*
Runcina sp. 20	*R. hornae*	Roses, Spain	MNCN 15.05/90662	*	*	*
Runcina sp. 20	*R. hornae*	Roses, Spain	MNCN 15.05/90663	*	*	*
Runcina sp. 21		Zut Island, Croatia	Pending number	*	*	*
Runcina sp. 22		Zut Island, Croatia	Pending number	*	*	*
Runcina sp. 22		Zut Island, Croatia	Pending number	*	*	*
Runcina sp. 24	*R. hornae*	Roses, Spain	MNCN 15.05/90664	*	*	*
Runcina sp. 26	*R. avellana*	Roses, Spain	MNCN 15.05/90653	*	*	*
Runcina sp. 29		Ugljan Island loc. Sv. Kuzman I Damjan (HR)	Pending number	*	*	*

Table 1. Continued.

Species Preliminary Ids	Revised Ids	Locality	Voucher no.	16S	COI	H3
Runcina sp. 29		Ugljan Island loc. Sv. Kuzman I Damjan (HR)	Pending number	*	*	*
Runcina sp. 29		Ugljan Island loc. Sv. Kuzman I Damjan (HR)	Pending number	*	*	*
Runcina sp. 30		Iz Island/Parda (HR)	Pending number	*	-	*
Runcina sp. 31		Es Caials (SP)	Pending number	*	*	*
Runcina sp. 33	*R. hornae*	Port Lligant (SP)	MNCN 15.05/90665	*	-	*
Runcina sp. 34	*R. adriatica*	Port Lligant (SP)	MNCN 15.05/90421	*	-	*
Runcina ferruginea		Banyuls-sur-Mer, France	ZSMMOL 20142074	-	*	*
Runcinida marisae Chernyshev, 1998		Vostok Bay, Rusia	-	-	KP313667	-
Runcinida valentinae Chernyshev, 2006		Iturup Island, Rusia	-	-	KP313666	-
Runcinida sp. 1		Cap Ferret, France	MNCN 15.05/90670	*	*	*
Runcinid sp. 1		Alagoas, Brasil	MZUSP103407	*	*	-
Lapinura sp. 1		Maui, Hawaii	MNCN 15.05/90671	*	*	*
Runcinid sp. 2		Quirimbas, Mozambique	ZMBN105119	-	*	*
Aplysia dactylomela Rang, 1828		Cape Verde (GB)	NHMUK 20030795/ 20030796	KJ022921	KF992168	KJ022798
Micromelo undatus (Bruguière, 1792)		Tenerife, Canary Island (GB)	NHMUK 20030800	KJ022944	DQ974653	KJ022778

Table 2. Uncorrected *p*-distances based on COI sequences within the order Runcinida. Roman numbers are based on species delimitation groups represented in Fig. 1.

	III	IV–V	VI–XXIII	XXIV	XXV	XXVI	XXVII	XXVIII
IV–V	19.3–20.7	15.8						
VI–XXIII	12.2–18.7	16.1–20.3						
XXIV	18.3–18.5	18.8–21	14.4–19.9	0.2				
XXV	16.9	19.5–20.5	15–21.1	17.1–17.8				
XXVI	17.5	19.5–19.9	15–20.2	15.3–15.5	13.1			
XXVII	16.9	18.3–20.1	16.6–20.9	16.3–16.4	13	15.7		
XXVIII	21	20.5–21.4	17.8–23.1	20.7–20.9	18.8	18.1	19.6	
XXIX–XXXI	19.3–20.7	17.1–20.7	16–22	16.9–17.5	16.2–17.4	18.1–19	18.5–19.3	19.1–19.8

Table 4. Differences between *Runcina coronata*, *Runcina caletae* sp. nov., *Runcina flavonigra* sp. nov. and *Runcina tingitana* sp. nov. Data after Quatrefages (1844), Vayssière (1883), García *et al*. (1986), Cervera *et al*. (1991), Schmekel and Cappellato (2002) and present study.

	Runcina coronata	*Runcina caletae* sp. nov.
Colour pattern	General ground colour dark brown. Dark brown on the central zone between the eyes and pale fawn on the sides. White chalk or yellowish spots all over the body. White semicircles bands behind the eyes and anterior to notum end. White spots on the surface of the foot. Dark longitudinal band on the dorsal surface of the foot.	Ground colour translucent pale fawn or yellowish. Tiny white, black and yellow spots all over the body. White spots on the lobes of the head and on the anterior ventral surface of the foot. Triangular white patches behind the eyes. White semicircle anterior to notum end. Small black dots forming two longitudinal lines on the head. Black dots posterior to white semicircle. Dark longitudinal band on the dorsal surface of the foot.
Gills	Three rounded gills, the largest middle one divided into two arcs. Yellowish with brown margins.	Three rounded gills. Yellowish with slightly brown margins.
Shell	Absent	Absent
Radular formulae	19 x 1.1.1	13 x 1.1.1
Radular teeth	Rachidian tooth bilobed. Two pads with 9–11 short denticles. Three or four more developed and thick alternate with smaller and thinner ones. Small depression between pads; denticle may be present in some rows. Lateral teeth denticulate, elongate and curved like a swan neck.	Rachidian tooth bilobed. Two pads with 7–8 long, pointed denticles stalactite shaped. Regular size, decreasing towards to the middle of the tooth. Depression between pads absent. Lateral teeth denticulate, elongated hooked shape with 33–36 long, pointed denticles.
Gizzard plate	10 crests	7–10 crests

Table 4. (Continued)

	Runcina flavonigra sp. nov.	*Runcina tingitana* sp. nov.
Colour pattern	Ground colour translucent pale fawn or yellowish. Digestive gland brownish. White spots on central zone of the notum, behind eyes forming triangular patches and anterior to notum end. Maybe absent. Black spots on the notum. Dark longitudinal band on the dorsal surface of the foot.	Ground colour light brown. Digestive gland visible (brownish colour). White spots behind eyes and anterior to notum end forming a triangle. Yellowish dots on the middle of notum. Dark small spots may be present in the white spots zones. Dark band on the dorsal surface of the foot
Gills	Four rounded and relatively large gills. Yellowish with slightly brown margins.	Two rounded gills. Light brown colour.
Shell	Absent	Absent
Radular formulae	12–13 x 1.1.1	12–14 x 1.1.1
Radular teeth	Rachidian tooth bilobed. Two pads each with 10–11 long, slender, pointed denticles. Small depression between pads apparently bearing a tiny denticle. Lateral teeth denticulate, elongate and hooked shape with 35–36 long and pointed denticles.	Rachidian tooth bilobed. Two pads with 7–10 short triangular denticles. The third or fourth denticle of each pad more developed. Small depression between pads; denticle absent. Lateral teeth denticulate, elongated, hooked shape with 34–37 long, thin, pointed denticles.
Gizzard plate	7–9 crests	8 crests

SUPPLEMENTARY FIGURES

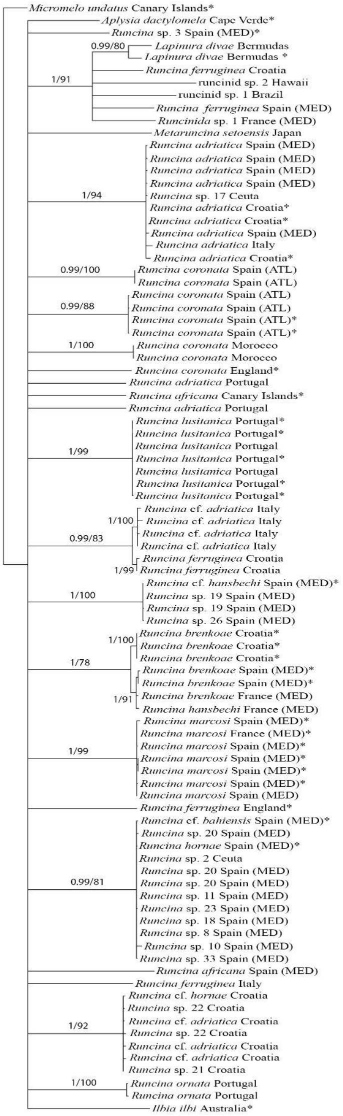

Figure S1. Phylogenetic hypothesis based on BI of the 16S gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

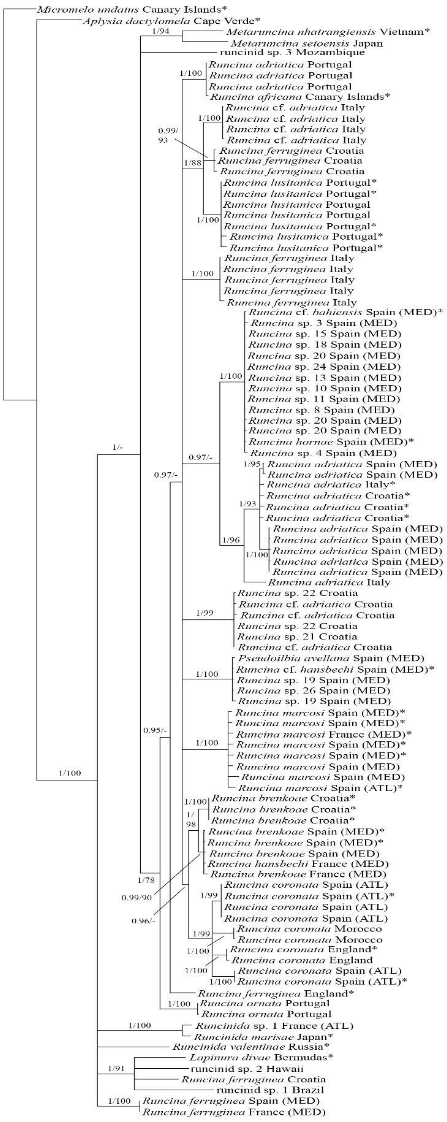

Figure S2. Phylogenetic hypothesis based on BI of the COI gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

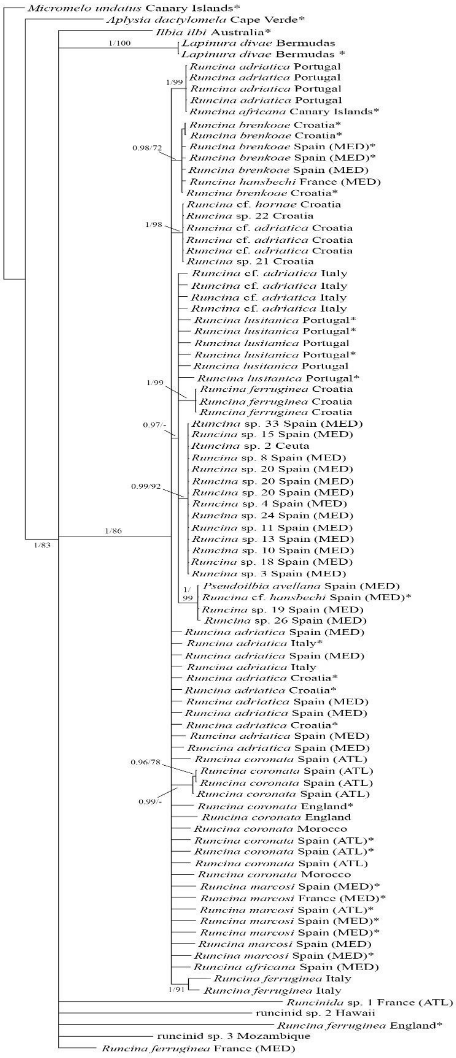

Figure S3. Phylogenetic hypothesis based on BI of the H3 gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

Capítulo 2

To be or not to be? What molecules say about *Runcina brenkoae* Thompson, 1980
(Gastropoda: Heterobranchia: Runcinida)

Araujo A.K, Pola M., Malaquias M.A.E., Cervera J.L. (2019)
Scientia Marina 83 (3). doi: 10.3989/scimar.04907.07A

(VER ANEXO I)

To be or not to be? What molecules say about *Runcina brenkoae* Thompson, 1980 (Gastropoda: Heterobranchia: Runcinida)

ABSTRACT

Runcina brenkoae Thompson, 1980, described from northern Adriatic Sea (Mediterranean Sea), show a characteristic pattern of anastomosing black blotches, red-brown colour and white chalk spots on the cephalic zone. However, this species has been described to display chromatic variability questioning the true identity and geographic distribution of the species. The molecular results in the Chapter I showed that not every specimen with a pattern of anastomosing black blotches belongs to *R. brenkoae*, revealing that species as a species complex. In this chapter, we investigate the "*R. brenkoae*" species complex following an integrative approach combining multi-locus molecular phylogenetics and morpho-anatomical characters, based on specimens from northern Adriatic Sea, western Mediterranean Sea and the southwestern Iberian coastline of Portugal and Spain. As result, we described three different species belonging to "*R. brenkoae*" species complex: *R. brenkoae* proper, R. *marcosi* sp. nov. and *R. lusitanica* sp. nov.

INTRODUCTION

Within the family Runcinidae, *Runcina* is the most species-rich genus, with 38 valid species of which 29 occur in European waters (Cervera *et al*. 2004, Schmekel and Cappellato, 2002, Ortea *et al*. 2015). The small size of these animals and the fact that most species have dark, dull cryptic colour patterns, render the runcinids difficult to be detected and identified.

One of the taxonomically difficult species of the European fauna is *Runcina brenkoae*, Thompson, 1980, which together with *Runcina adriatica* Thompson, 1980 and *Runcina zavodniki* Thompson, 1980 have been described from the Adriatic Sea. *Runcina brenkoae* is characterized by an elongated body with a characteristic pattern of anastomosing black blotches, a red-brown ground colour, clusters of chalk white spots on both sides of the head behind the eyes, and presence of two gills. However, Thompson and Brodie (1988) referred to specimens of *R. brenkoae* collected near Rovinj (Croatia), the type locality, which depicted several differences in respect to the original description, like the presence of a developed crest, a pale fawn ground colour and the absence of white spots. Nevertheless, the specimens possessed key features of the species such as the anastomosing black blotches and presence of only two gills. Schmekel and Cappellato (2002) reported for the first time the species outside the Adriatic Sea in Banuyls-sur-Mer (French Mediterranean coast) and Ballesteros *et al.* (2016) cited *R. brenkoae* in Catalonia (Spanish northeastern coast).

The use of integrative taxonomic approaches and in particular of molecular phylogenetics have revealed the existence of numerous species complexes and contributed to the discovery of unknown species among heterobranch sea slugs (Padula *et al*. 2014, Austin *et al.*, 2018, Krug *et al*. 2018; among others). In Chapter I, our phylogenetic analyses revelead *R. brenkoae* as another example of cryptic diversity masked under a single species name. Therefore, in this chapter, we follow an integrative approach combining multi-locus molecular phylogenetics and morpho-anatomical characters to investigate the taxonomic status of *R. brenkoae*, based on specimens from the central and western Mediterranean Sea and the southern Iberian coastline of Portugal and Spain.

MATERIALS AND METHODS

DNA extraction, amplification and sequencing

All specimens used in this study were those obtained in Chapter I and from Genbank (Table 1). *Micromelo undatus* (Bruguière, 1792) was used to root the tree as outgroup taxon. Tissue samples were taken from the foot and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). DNA extraction, amplification and sequencing follow Chapter I of this Thesis.

Specimens from the type locality, Rovinj (Croatia) could not be obtained, but we sequenced specimens from nearby localities, Nin and Split mean distance 218.77 km away to south from Rovinj.

Phylogenetic analyses

Sequences were edited in Genious v10.2.3 (Drummond *et al*. 2009) and aligned using MAFFT (Katoh *et al*., 2009) implemented in Geneious v10.2.3 (Drummond *et al*. 2009) with the default settings (Auto [FFT-NS-1, FFT-NS-2, FFT-NS-i or L-INS-i; depends on data size]). Sequences from the protein-coding genes COI and H3 were translated into amino acids to check for stop-codons. Hypervariable regions of the 16S alignment where homology could not be confidently established were removed using in Gblocks under relaxed settings (Tavalera and Castresana, 2007). Nevertheless, analyses including and excluding these regions provided similar results. Therefore, final analyses were performed including all bases. Sequences of the COI, 16S and H3 genes were trimmed to 658, 457 and 328 nucleotides, respectively. All three genes were concatenated using Mesquite v3.2 (Maddison and Maddison, 2018), resulting in a final dataset of 1443 base pairs. Single gene and a concatenated (H3+COI+16S) analyses were performed. Saturation for the first, second and third codon positions of the COI and H3 genes were calculated in MEGA v7.0 (Kumar *et al*., 2016).

The best-fit evolutionary model for each gene was determined in jModeltest v2.1.6 (Guindon and Gascuel, 2003; Darriba *et al*., 2012), under the Akaike information criterion (AIC) (Akaike, 1974). The GTR + G + I model was selected for the COI and H3 genes, and GTR + G for the 16S gene. Bayesian inference (BI) analyses were performed in MrBayes v. 3.2.1 (Ronquist and Huelsenbeck, 2003) with a random starting tree and two parallel runs of 10^7 generations. Convergence was checked in TRACER v1.7.1 (Rambaut *et al*., 2018) with a burn-in of 25%. Nodes with a posterior probability (PP) ≥ 0.95 (Alfaro, Zoller and Lutzoni, 2003) were considered well supported and discussed. Maximum likelihood (ML) analysis was executed using RAxML v8 (Stamatakis, 2014) and node support was assessed with nonparametric bootstrapping (BS) with 5000 replicates. Nodes with bootstrap values (BS) ≥ 70 (Hillis and Bull, 1993) were considered significant and were discussed. Both BI and ML trees were visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Minimum and maximum pairwise uncorrected *p*-distances of COI within and between species were calculated in MEGA v7.0 using all sequences available. (Kumar *et al*., 2016).

Species delimitation analyses

The Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al*., 2012) and the Bayesian Poisson tree processes (bPTP) (Zhang *et al*., 2013) were used to aid delimiting species. For the ABGD we used the alignment from the fast-evolving COI gene with default settings ($P_{\mathrm{min}} = 0.001$, $P_{\mathrm{max}} = 0.1$, Steps = 10, $X = 1.2$, Nb bins = 20), under the three models of evolution available, namely Jukes-Cantor (JC69), Kimura (K80) and Simple Distance. The bPTP analysis is an updated version of the original maximum likelihood PTP (modelling speciation in terms of the number of substitutions), which adds Bayesian support values to delimit species. The bPTP analyses were run with the COI and 16S trees using the webserver (https://species.h-its.org/ptp/) (Zhang *et al*., 2013).

Morphology

To complete and compare the results obtained by molecular phylogenetics and species delimitation analyses, specimens previously identified as *Runcina brenkoae* and *Runcina* sp. from Croatia (Adriatic) (3), Catalonia (Mediterranean, Spain) (6), Cadiz (Atlantic, Spain) (1) and Algarve (Atlantic, Portugal) (5), and one specimen early identified as *R. adriatica* from Banyuls-sur-Mer (France) were studied for their morpho-anatomy. Animals were dorsally dissected and the buccal bulbs were extracted and dissolved in a solution of 10% sodium hydroxide to expose the radula. The radulae and gizzard plates were then immersed in water, dried and mounted for scanning electron microscopy (SEM) with a Nova NanoSEM 450 available at the University of Cadiz (Cadiz, Spain). The reproductive system was examined and drawn using a dissecting microscope with the aid of a *camera lucida*.

RESULTS

Phylogenetic analyses

The concatenated (H3+COI+16S) tree provided better resolution than the individual gene analyses (Figs 1, S1, S2 and S3). No saturation was observed, not even in the third codon position. Both BI and ML analyses support the monophyly of the genus *Runcina* (PP = 1; BS = 100) and showed *L. divae* to be its sister lineage (PP = 0.98; BS = 86). The species *Ilbia ilbi* was rendered sister to the *Lapinura* + *Runcina* clade (PP = 1; BS = 100). In the *Runcina* clade the species *R. ferruginea* was rendered sister to a sub-clade containing all remaining species (PP = 1; BS = 80). The specimens previously identified as *R. brenkoae* split into three subclades all with maximum support (PP = 1; BS = 100). The first clade (Group A) includes specimens from Portugal; the second clade (Group B) contains one specimen previously identified as *Runcina adriatica* from France (Mediterranean), and specimens from Spain (Atlantic and Mediterranean); and the third clade (Group C) includes specimens from Croatia and Spain (Mediterranean) (Fig. 1).

Species delimitation analyses

The ABGD analysis of the COI sequences with all three models of evolution resulted in 11 groups with three of them corresponding to the same *R. brenkoae* groups A, B and C recovered in the BI and ML analyses (Fig 1A). However, the recursive partition, at lower values of prior intraspecific divergence (P), recovered seven groups for the "*R. brenkoae* complex", separating specimens from Group A and C into two distinct groups each, and specimens from Group B into three distinct groups (not shown).

Regarding the COI uncorrected *p*-distances, the minimum distance between Groups A and B was 11.7%; between Groups A and C is 9.6%; and between Groups B and C was 10.4%. The maximum distance within specimens of Group A was 0%, within Group B 4%, and within Group C was 4.6% (Table 2). Between species in the genus *Runcina* the COI uncorrected *p*-distances ranged from 9.3% to 15.1%, while between the genera *Runcina* and *Lapinura* ranged from 16.3% to 20.7%. No COI gene sequences from *Ilbia ilbi* were available for this analysis. The results obtained with the bPTP analysis were congruent with the ABGD output, by suggesting the same three groups of *Runcina brenkoae* (Fig. 1B).

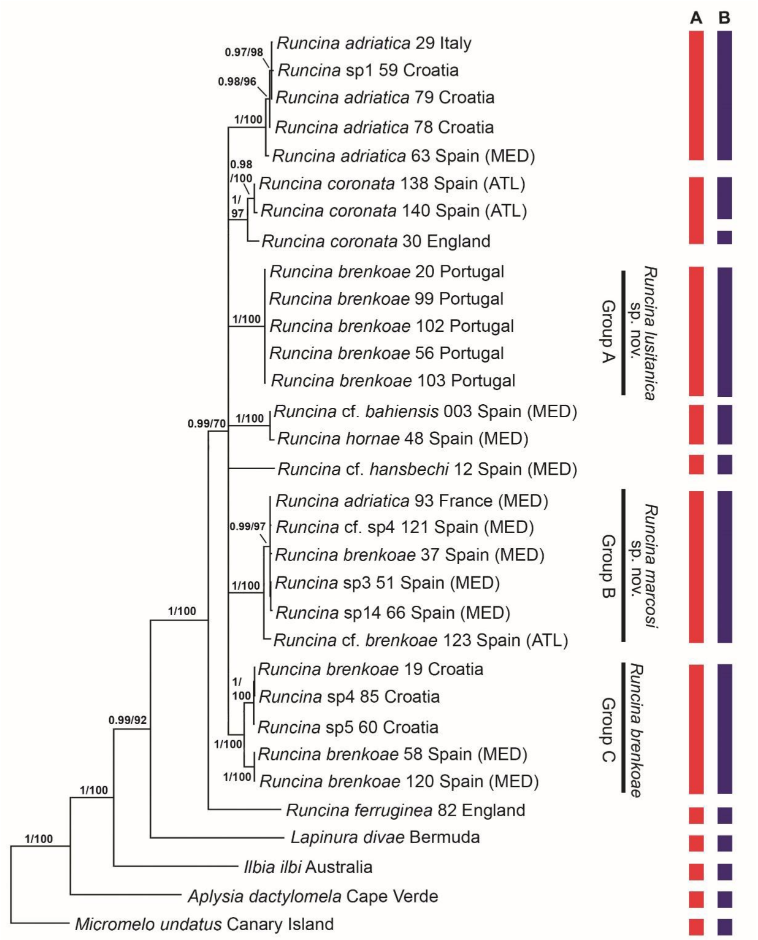

Figure 1. Phylogenetic hypothesis of "*Runcina brenkoae*" complex based on the combined dataset (H3+COI+16S) inferred by Bayesian analysis. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Numbers after the sequences name refers to individual specimen number. Abbreviations: ATL, Atlantic Ocean; MED, Mediterranean Sea. (**A**) ABGD based on the COI data set. (**B**) bPTP result based on the COI and 16S data sets

Table 2. Uncorrected *p*-distances based on COI sequences.

	Distance between groups (%)		Distance within groups (%)
	Group A	Group B	
Group A			0.0
Group B	11.7 – 12.0		0.0 – 4.0
Group C	9.6 – 11.6	10.3 – 11.5	0.0 – 4.6

The molecular results support the occurrence of three species under the name *Runcina brenkoae* and this is backed by morphological differences across specimens from the three *R. brenkoae*-clades (see theme Systematic description). Therefore, we present below a redescription of *R. brenkoae* and describe two new species. A comparison between these three species is shown in Table 3.

Systematic description

Family RUNCINIDAE H. Adams and A. Adams, 1854
Runcina Forbes in Forbes and Hanley, 1851

Runcina brenkoae Thompson, 1980
(Figs 2; 5A–C; 6A, D)

Runcina brenkoae Thompson, 1980: 156, fig. 1C. Thompson, T.E. and Brodie, G. 1988: 340, fig. 1D. Schmekel, L. and Capellato, D. 2001: 144, Pl. I g; 145, Pl. II k; and 148, Pl. III a, b; Schmekel, L. and Capellato, D., 2002: 98, Pl. VI a – c. Ballesteros *et al*., 2016: 4, fig. 7A.

Type material: Holotype (NHMUK 197913W) Natural History Museum, London, UK (not studied because the material is only available as micro-slide preparations).
Type locality: Rovinj, Croatia.
Examined material: MNCN 15.05/88086, Split, Croatia, 03 Aug 2014, 1.5 mm in length preserved, depth 1 m. Found washing *Posidonia* (dissected and sequenced). MNCN 15.05/88087, Roses, Catalonia, Spain, coll. Marina Poddubetskaia, 08 Aug 2016, 1 mm in length preserved, depth 8 mm. Found on *Posidonia* (dissected, sequenced). MNCN 15.05/88088, Nin, Croatia, coll. Alen Petani, 04 Apr 2017, 3.5 mm in length preserved, depth

0.5-1 m (dissected and sequenced). MNCN 15.05/88090, Roses, Catalonia, Spain, coll. Marina Poddubetskaia, 19 July 2017, 1 mm in length preserved, depth 9 m (sequenced). MNCN 15.05/88089, Nin, Croatia, coll. Alen Petani, 26 Dic 2017, 1.5 mm in length preserved, depth 0-1 m (sequenced).

External morphology (Fig. 2): Body moderately elongated and tapered. Notum smooth. Foot as wide as notum, showing a developed median pallial crest. Ground colour of body red-brown, sometimes translucent pale fawn bearing a pattern of anastomosing dark blotches on notum, margin, and sole of foot. Eyes difficult to discern. Chalk white spots all over body, more concentrated on margin of tail, both sides of head behind eyes and on metapodium in front of dark band. Some specimens with small red spots on margin of tail and surface of metapodium. The slugs have strike a longitudinal band of dark brown or red wine on the surface of the metapodium. Two equal sized translucent gills with white spots bearing pinnules on right posterior side of body. Anal pore situated beneath gills.

Internal anatomy (Figs 5A–C; 6A, D): Radular formulae 20 x 1.1.1 (MNCN 15.05/88086, MNCN 15.05/88088). Rachidian tooth boomerang shaped with long, smooth lateral wings on each side. Central part of rachidian tooth bilobed; masticatory edge contains a pair of rounded pads with a cockle-shaped, each pad possesses 8–10 denticles. Median deep and broad depression present between the pads; a small denticle may be present (Fig. 5A). Lateral teeth smooth, elongate and curved like a swan neck (Fig. 5B). Triangular jaws present. Four gizzard plates with 5–7 lamellae (Fig. 5C). Shell absent. Reproductive system monoaulic. Female gland mass slightly divided into two lobes. Common genital duct connecting the female gland to the exterior on the posterior right side of the body. Bursa copulatrix absent. Female gland placed on the posterior right side of digestive gland (Fig. 6A). Male copulatory organ opens to the right of the mouth. Short and unarmed penial papilla projects into the atrium. Prostate gland long and cylindrical. Slender seminal vesicle with half size of prostate gland (Fig. 6D).

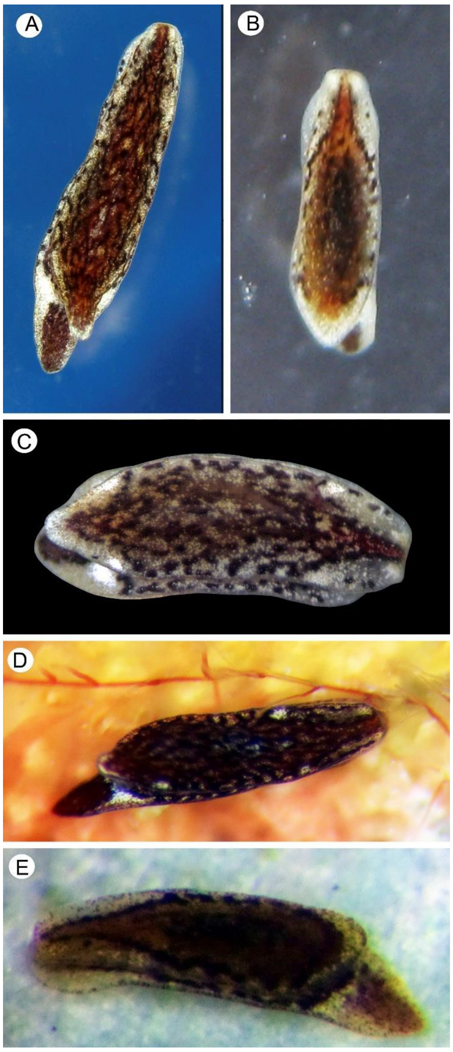

Figure 2. Living animals of *Runcina brenkoae*. **A** MNCN 15.05/88086, **B** MNCN 15.05/88089, **C** MNCN 15.05/88088. Specimens from Croatia (Adriatic Sea) (photos Alen Petani). **D** MNCN 15.05/88087, **E** MNCN 15.05/88090. Specimens from Catalonia, Spain (Mediterranean) (photos Marina Poddubetskaia).

Runcina lusitanica sp. nov.

(Figs 3; 5D–F; 6B, E)

Examined material: Holotype: MNCN 15.05/200065, Near Faro, Algarve, Portugal, coll. Jorge Antonio Domínguez Godino, May 2015, 4 mm in length preserved (dissected and sequenced). Paratypes: MNCN 15.05/88091, Near Faro, Algarve, Portugal, coll. Jorge Antonio Domínguez Godino, May 2015, 5 mm in length preserved (dissected and sequenced). MNCN 15.05/88092, Near Faro, Algarve, Portugal, coll. Jorge Antonio Domínguez Godino, May 2015, 5 mm in length preserved (dissected and sequenced). MNCN 15.05/88093, Near Faro, Algarve, Portugal, coll. Jorge Antonio Domínguez Godino, May 2015, 4.5 mm in length preserved (dissected and sequenced). MNCN 15.05/88094, Near Faro, Algarve, Portugal, coll. Jorge Antonio Domínguez Godino, May 2015, 4.5 mm in length preserved (dissected and sequenced).
Etymology: Lusitania was the name of a Roman province in the west of the Iberian Peninsula that occupied much of what now is Portugal.

External morphology (Fig. 3): Body elongated and moderately broad. Notum smooth. Foot as wide as notum. Posterior part of notum rounded without pallial crest. Ground colour of body brown and translucent yellowish bearing a pattern of anastomosing dark blotches on notum and margin of foot. Some specimens present a large pale fawn patch on the posterior part of head and notum. Eyes not visible. White spots in some specimens. Longitudinal band, sometimes wide, of dark brown on surface of metapodium. Two big yellowish gills with dark spots bearing irregular pinnules on right posterior side of body. Upper gill unipinnate and the most ventral bipinnate. Anal pore situated beneath gills.

Internal anatomy (Figs 5D–F; 6B, E): Radular formulae 25 x 1.1.1 (MNCN 15.05/88092) and 29 x 1.1.1 (MNCN 15.05/88093). Rachidian tooth boomerang shaped with one long and smooth lateral wings on each side. Central part of rachidian tooth bilobed; masticatory edge contains a pair of flat pads comb shaped, each one possessing 10–12 denticles. Median deep and broad depression is present between the pads; a small denticle present (Fig. 5D). Lateral teeth smooth, elongate and curved like a swan neck (Fig. 5E). Triangular jaws present. Four gizzard plates with 10–11 lamellae (Fig. 5F). Shell absent. Reproductive system monoaulic. Female gland mass divided into two lobes, located on right side and behind the digestive gland. Bursa copulatrix absent. Common genital duct opening to exterior on posterior right side of body (Fig. 6B). Male copulatory organ comprises a relatively large atrium, which opens on right side next to mouth. Inside atrium projects short, unarmed, conical penial papilla. Long and cylindrical prostate gland. Elongated and convoluted seminal vesicle (Fig. 6E).

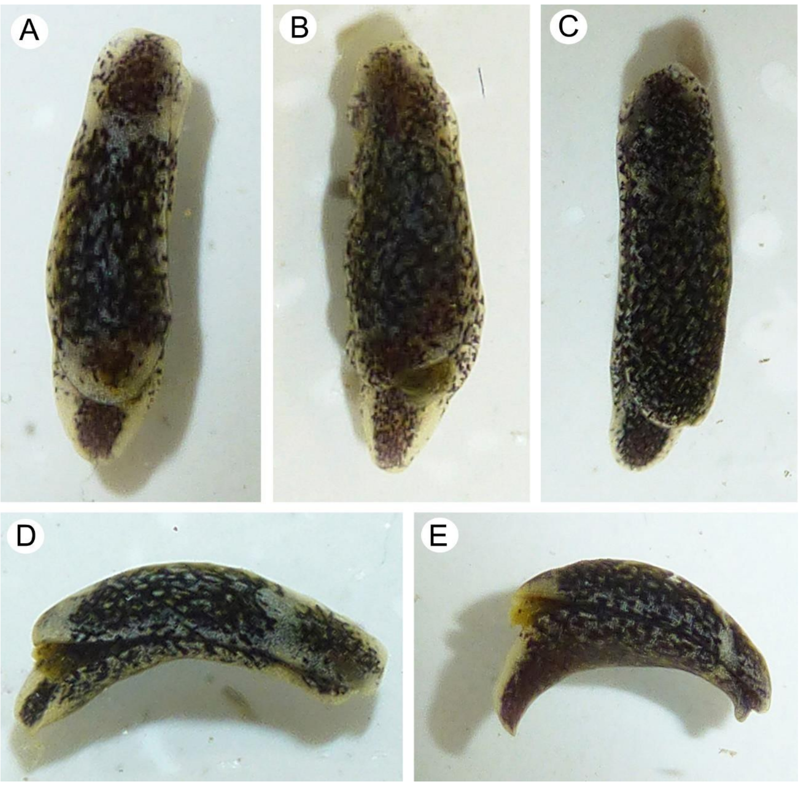

Figure 3. Living animals of *Runcina lusitanica* sp. nov. (south coast of Portugal). A MNCN 15.05/88092, **D** MNCN 15.05/200065, **E** MNCN 15.05/88094. Specimens showing the absence of dark blotches on the posterior part of the head and notum. **B** MNCN 15.05/88091, **C** MNCN 15.05/88093. Specimens with dark blotches covering all the notum.

Runcina marcosi **sp. nov.**

(Figs 4; 5G–I; 6C, F, G)

Examined material: Holotype: MNCN 15.05/200066, La Caleta (Cadiz), Andalusia, southwestern, Spain, coll. Josep Romà, 17 May 2015, 2.5 mm in length preserved, depth 0.5 – 1 m. Found on samples of the brown algae *Halopteris scoparia* (dissected and sequenced). Paratypes: ZSM MOL 201442089, Banyuls-sur-Mer, France (Mediterranean), coll. Bastian Brenzinger and Timea Neusser, July 2nd 2014, 1.3 mm in length preserved (sequenced). MNCN 15.05/88095, Mataro, Catalonia, northeastern, Spain, coll. Manuel Ballesteros, 22 Sep. 2015, 1 mm in length preserved. Found on green algae (dissected and sequenced). MNCN 15.05/88096, Roses, Catalonia, northeastern, Spain, coll. Carles Galià, 29 May 2017, 1 mm in length preserved. Found on roots of seagrass *Posidonia oceanica* (dissected and sequenced). MNCN 15.05/88098, Roses, Catalonia, northeastern, Spain, coll. Marina Poddubetskaia, 30 Aug. 2017, 1.5 mm in length preserved (dissected and sequenced). MNCN 15.05/88097, Roses, Catalonia, northeastern, Spain, coll. Marina Poddubetskaia, 19 July 2018, 2 mm in length preserved, depth 4 m (sequenced).

Etymology: This species is dedicated to Marcos Martínez Vazquez, husband of the first author, for all his help, enthusiasm, and support, during the course of this work.

External morphology (Fig. 4): Body moderately elongated. Notum smooth. Foot as wide as notum. Some specimens show developed median pallial crest. Ground colour of body red-brown or translucent pale fawn bearing a pattern of anastomosing dark or reddish blotches on notum, margin of foot and metapodium. Eyes difficult to discern. White spots all over the body. Longitudinal band of dark brown or wine red on the surface of metapodium. Two translucent gills bearing regular pinnules on right posterior side of body. Upper gill unipinnate and the most ventral bipinnate. Anal pore situated beneath gills.

Internal anatomy (Figs 5G–I; 6C, F, G): Radular formulae 10 x 1.1.1 (MNCN 15.05/88097) and 13 x 1.1.1 (MNCN 15.05/88095). Rachidian tooth boomerang shaped with long and smooth lateral wings on each side. Central part of rachidian tooth bilobed; masticatory edge contains a pair of flat pads comb shaped, each one possessing 10–11 denticles. Median deep and broad depression present between the pads; small denticle absent (Fig. 5G). Lateral teeth smooth,

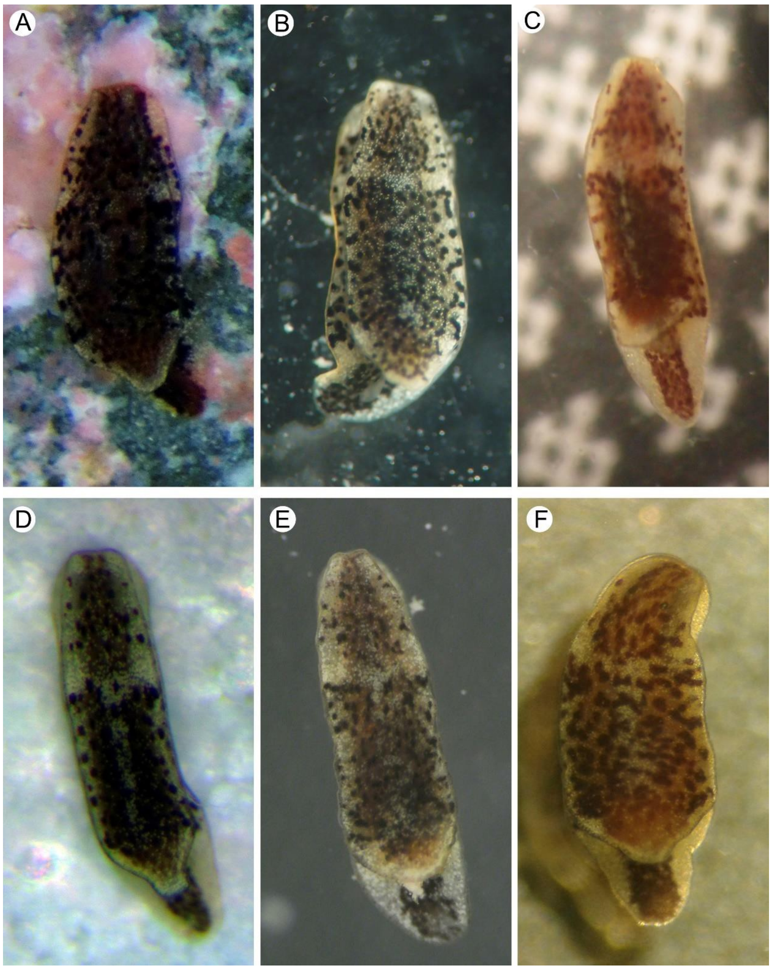

Figure 4. Living animals of *Runcina marcosi* sp. nov. **A** MNCN 15.05/88098, **B** MNCN 15.05/88095, **C** MNCN 15.05/88096, **D** MNCN 15.05/88097. Specimens from Catalonia, Spain (Mediterranean) (photos Marina Poddubetskaia, Ana Karla Araujo, Carlès Galiá). **E** ZSM MOL 201442089. Specimen from Banyuls-sur-Mer, France (Mediterranean) (photo Bastian Brenzinger). **F** MNCN 15.05/200066. Specimen from Cadiz, Spain (Atlantic) (photo Ana Karla Araujo).

elongate and curved like a swan neck (Fig. 5H). Triangular jaws present. Four gizzard plates with 7–8 lamellae (Fig. 5I). Shell absent. Reproductive system monoaulic. Female gland mass placed on right side and behind the digestive gland. Divided into two lobes, may be albumen and mucous glands. Long common genital duct connects the female gland to exterior on posterior right side of body. Bursa copulatrix absent (Fig. 6C). Elongated and cylindrical male copulatory organ. Atrium opens to the right side of mouth. Short and unarmed penial papilla projects into the atrium. Cylindrical prostate gland. Slender seminal vesicle with half size of prostate gland (Fig. 6F, G).

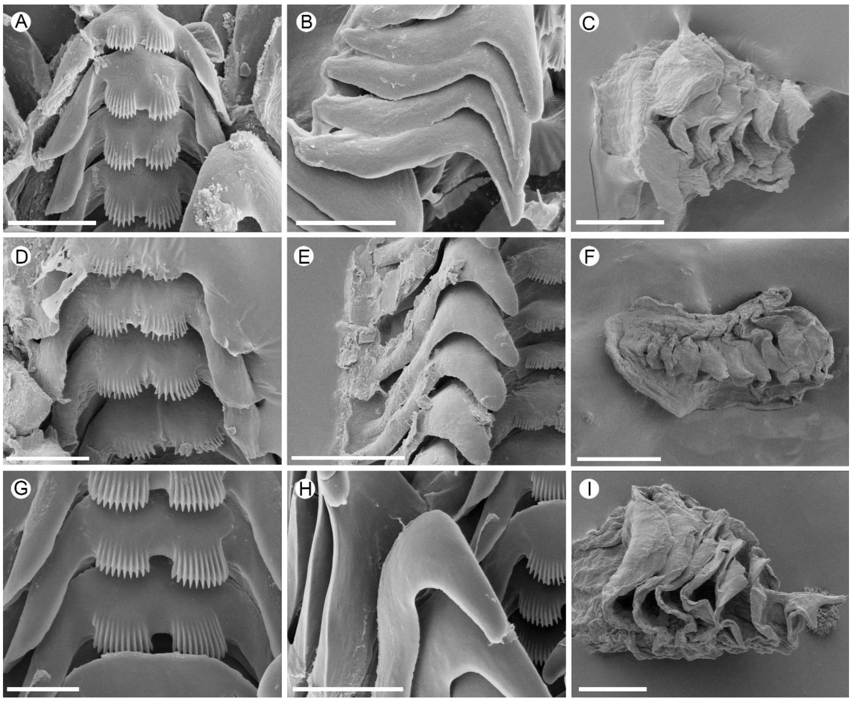

Figure 5. Scanning electron micrographs of jaw and radula of *Runcina* species. **A, B, C**. *R. brenkoae* (MNCN 15.05/88086, MNCN 15.05/88088). **A**. Radular teeth (MNCN 15.05/88088). **B**. Lateral teeth (MNCN 15.05/88086). **C**. Gizzard plate (MNCN 15.05/88088). **D, E, F**. *R. lusitanica* sp. nov. (MNCN 15.05/88093) **D**. Radular teeth. **E.** Lateral teeth. **F**. Gizzard plate **G, H, I**. *R. marcosi* sp. nov. (MNCN 15.05/88095, MNCN 15.05/88097). **G**. Radular teeth (MNCN 15.05/88095). **H**. Lateral teeth (MNCN 15.05/88095). **I**. Gizzard plate (MNCN 15.05/88097). Scale bars: **A, B, E, H** = 10 µm; **C** = 50 µm; **D, I** = 20 µm; **F** = 100 µm; **G** = 5 µm

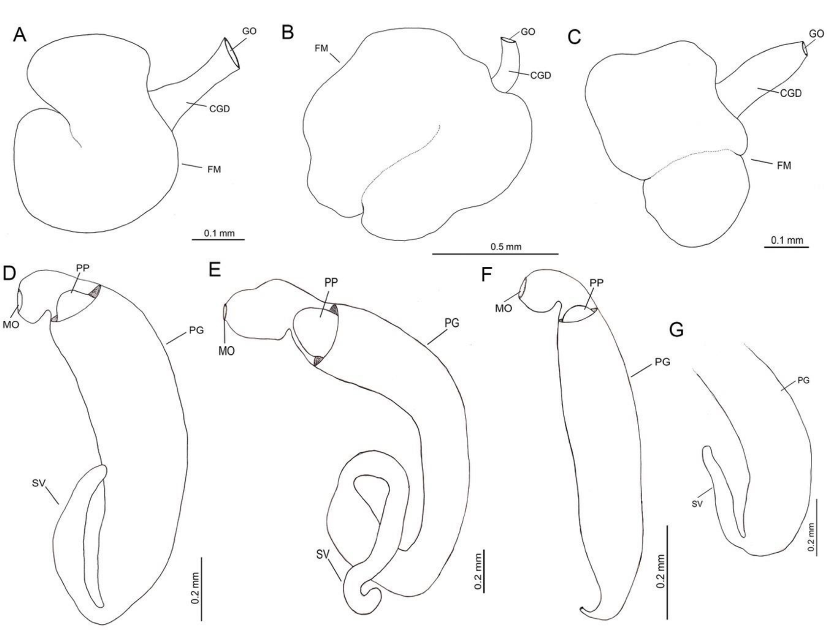

Figure 6. Reproductive system of *Runcina* species. Top row female part (A, B, C); lower row male part (D, E, F, G). **A, D**. *R. brenkoae*, Croatia (MNCN 15.05/88088). **B, E**. *R. lusitanica* sp. nov., Portugal (MNCN 15.05/88093). **C, G**. *R. marcosi* sp. nov., Cadiz, Spain (MNCN 15.05/200066); male copulatoryorgan damaged without penial papilla, but showing seminal vesicle. **F**. *R. marcosi* sp. nov., Catalonia, Spain (MNCN 15.05/88097); male copulatory organ damaged without seminal vesicle. Abbreviations: fm, female mass; cgd, common genital duct; go, gonopore; mo, male opening; pp, penial papilla; pg, prostate gland; sv, seminal vesicle.

DISCUSSION

Recent molecular studies on heterobranch sea slugs, mostly on nudibranchs, have demonstrated the existence of many complexes of cryptic species (Austin *et al.*, 2018, Layton *et al.*, 2018, Korshunova *et al.*, 2019; among many others). Up to now, most studies related to the order Runcinida have focused only on morphological aspects addressed to identify and describe new species and genera (Cervera *et al.*, 1991, Chernyshev, 2006, Moro and Ortea, 2015). The first phylogenetic analysis focus on the order Runcinida (see Chapter I) revealed *R. brenkoae* as species complex. Our study recognized three distinct species within this complex, namely the proper *R. brenkoae* Thompson, 1980, and two new species here described as *R. marcosi* sp. nov. and *R. lusitanica* sp. nov.

Externally, all species of this complex are similar in colour, but *R. marcosi* sp. nov., despite its chromatic variability, has a characteristic concentration of white spots on the anterior part of the body forming a "necklace" (Figs 2–4; Table 3). *R. brenkoae* is the only one among the three species of the complex with both gills unipinnate, whereas *R. lusitanica* sp. nov. and *R. marcosi* sp. nov. have one gill unipinnate and the other bipinnate. *R. lusitanica* sp. nov. reaches comparatively larger sizes (up to 5 mm in length in preserved animals), but chromatically overlaps with *R. brenkoae* (Figs 2, 3; Table 3). *R. marcosi* sp. nov. shows a remarkable chromatic variation (Fig. 4) and in fact, some individuals can be confused with *R. adriatica* which has chalk-white spots on the pallial crest and behind the eyes forming a "necklace" (Thompson, 1980, Thompson and Brodie, 1988). However, *R. adriatica* has three gills (two bipinnate and one unipinnate) and a higher number of radular rows (21 x 1.1.1) (Thompson, 1980).

Anatomically these species differ on subtle details of the radula and gizzard plates. The pads of the rachidian tooth are more oval in shape in *R. brenkoae* as observed by Schmekel and Cappellato (2001, 2002), whereas in *R. marcosi* sp. nov. and *R. lusitanica* sp. nov. these pads are more flattened. In *R. lusitanica* sp. nov. and *R. brenkoae*, a small denticle is present in the depression between the two pads, but it may be absent in some rows. The gizzard plates of *R. brenkoae* have 5–6 lamellae, while in *R. marcosi* sp. nov. and *R. lusitanica* sp. nov. they have 7–8 and 10–11 lamellae, respectively (Table 3).

The male copulatory organ of the runcinids has a penial papilla projecting into an atrium; a prostate gland, and a seminal vesicle (Vayssière, 1883, Kress, 1977, Burn and Thompson, 1998). In both *R. brenkoae* and *R. marcosi* sp. nov. the male copulatory organ does not differ much from each other (Fig. 6D, F, G). The prostate is more curved in *R. brenkoae* than *R. marcosi* sp. nov., and the seminal vesicle in *R. brenkoae* is more rounded in one of the sides. Thompson (1980) did not mention any aspect of the male organ of *R. brenkoae*, nor did Thompson and Brodie (1988). Schmekel and Cappellato (2002) only refered to the *R. brenkoae* copulatory organ being similar to that of *R. ferruginea*, which has the same basic anatomical structure as those species described here. In *R. lusitanica* sp. nov. the penial papilla is comparatively larger than in *R. brenkoae* and *R. marcosi* and the posterior end of the cylindrical prostate narrows slightly into a very long and twisted seminal vesicle, which is not present in *R. brenkoae* and *R. marcosi* sp. nov (Fig. 6D – G).

The female part of the reproductive system in runcinids consists of an albumen and mucous gland opening to the outside through a common genital duct (Vayssière, 1883, Kress, 1977, Burn and Thompson, 1998). Yet, the presence of an ampulla and bursa copulatrix have been described for the species *Runcina macfarlandi* (Gosliner, 1991), *R. coronata*, *Ilbia ilbi*, among others (Vayssière, 1883, Burn, 1963, Gosliner, 1991). All three species of the *R. brenkoae* complex have similar female glands and we could not recognize an ampulla and bursa copulatrix (Fig. 6A–C). In general, the female part of the reproductive system in runcinids is poorly studied and for example Thompson (1980), Thompson and Brodie (1988), and Schmekel and Cappellato (2002) never referred to it.

Our study suggests that the geographical distribution of *Runcina brenkoae* proper is restricted to the Adriatic Sea (Croatia) and to the western Mediterranean (Spain and France), where it overlaps with the species *R. marcosi* sp. nov. at least in northeastern Spain (Mediterranean Sea). Schmekel and Cappellato (2001, 2002) referred to its presence in Banyuls-sur-Mer (French Mediterranean coast) but their specimens were initially fixed in formalin (Ronald Janssen, pers. com., Senckenberg Research Institute and Natural History Museum) and could not be tested for DNA. Thus, under the present taxonomic scenario the identity of these samples remains doubtful. The species *R. lusitanica* sp. nov. is so far only known from the southern coast of Portugal. Concerning the species *R. marcosi* sp. nov., its distribution is restricted to southwestern Spain (Atlantic) and western Mediterranean (Spain and France).

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Table 1. List of specimens used for phylogenetic analysis. (*) New sequences generated for this study. Museum abbreviations: Museum Victoria collections (NMVF), University Museum of Bergen (ZMBN) Zoologische Staatssammlung München, Germany (ZSM), Museo Nacional de Ciencias Naturales (MNCN) and The Natural History Museum, London, United Kingdom (NHMUK).

Species	New taxonomic assignment after phylogenetic study	Locality	Voucher no.	H3	COI	16S
Ilbia ilbi Burn, 1963		Hamers Haven, Australia	NMVF234189	MK322992*	-	MK323019*
Lapinura divae (Ev. Marcus and Er. Marcus, 1963)		Bermuda (GB)	ZMBN 82997	KJ022893	KF992195	KJ022825
Runcina sp. 1	*Runcina adriatica* T. Thompson, 1980	Iz Island, Croatia	MNCN 15.05/88099	MK323015*	MK322986*	MK323036*
Runcina adriatica T. Thompson, 1980		Ugljan Island, Croatia	MNCN 15.05/88100	MK322996*	MK322967*	MK323022*
Runcina adriatica T.		Zut Island, Croatia	MNCN 15.05/88101	MK322997*	MK322968*	-
Runcina adriatica T.		Otranto, Italy	MNCN 15.05/88102	MK322993*	MK322966*	-
Runcina adriatica T.		Cadaqués, Spain	MNCN 15.05/88103	MK322994*	MK322987*	MK323020*
Runcina adriatica T.	***Runcina marcosi* sp. nov.**	Banyuls-sur-Mer, France	ZSM MOL 201442089	MK322995*	MK322991*	MK323021*
Runcina brenkoae T. Thompson, 1980		Split, Croatia	MNCN 15.05/88086	MK322998*	MK322969*	MK323023*
Runcina sp.4	***Runcina brenkoae***	Nin, Croatia	MNCN 15.05/88089	MK323017*	MK322972*	MK323038*
Runcina sp.5	***Runcina brenkoae***	Nin, Croatia	MNCN 15.05/88088	MK323018*	MK322971*	MK323039*

Table 1. Continued.

Species	New taxonomic assignment after phylogenetic study	Locality	Voucher no.	H3	COI	16S
Runcina brenkoae T.		Roses, Spain	MNCN 15.05/88087	MK323001*	MK322970*	MK323026*
Runcina brenkoae T.		Roses, Spain	MNCN 15.05/88090	MK323003*	MK322964*	-
Runcina brenkoae T.	***Runcina marcosi* sp. nov.**	Roses, Spain	MNCN 15.05/88098	MK323000*	MK322982*	MK323025*
Runcina sp.3	***Runcina marcosi* sp. nov.**	Mataro, Spain	MNCN 15.05/88095	MK323016*	MK322983*	MK323037*
Runcina cf. sp.4	***Runcina marcosi* sp. nov.**	Roses, Spain	MNCN 15.05/88097	MK323012*	MK322990*	-
Runcina sp.14	***Runcina marcosi* sp. nov.**	Roses, Spain	MNCN 15.05/88096	MK323013*	MK322984*	MK323034*
Runcina cf. *brenkoae* T.	***Runcina marcosi* sp. nov.**	Cadiz, Spain	MNCN 15.05/200066	MK323006*	MK322985*	-
Runcina brenkoae T.	***Runcina lusitanica* sp. nov.**	Faro, Portugal	MNCN 15.05/88091	MK322999*	MK322979*	MK323024*
Runcina brenkoae T.	***Runcina lusitanica* sp. nov.**	Faro, Portugal	MNCN 15.05/200065	MK323002*	MK322981*	-
Runcina brenkoae T.	***Runcina lusitanica* sp. nov.**	Faro, Portugal	MNCN 15.05/88093	MK323004*	MK322965*	-
Runcina brenkoae T.	***Runcina lusitanica* sp. nov.**	Faro, Portugal	MNCN 15.05/88092	MK323014*	MK322980*	MK323035*
Runcina brenkoae T.	***Runcina lusitanica* sp. nov.**	Faro, Portugal	MNCN 15.05/88094	-	MK322989*	MK323027*
Runcina cf. *bahiensis* Cervera, Garcia-Gomez and Garcia, 1991		Cadaqués, Spain	MNCN 15.05/88104	MK323005*	MK322988*	-

Table 1. Continued.

Species	New taxonomic assignment after phylogenetic study	Locality	Voucher no.	H3	COI	16S
Runcina coronata (Quatrefages, 1844)		Swanage, England	MNCN 15.05/88105	MK323010*	MK322976*	MK323031*
Runcina coronata		Cádiz, Spain	MNCN 15.05/88106	MN057641*	-	MN057638*
Runcina coronata		Cádiz, Spain	MNCN 15.05/88107	MN057642*	MN057640*	MN057639*
Runcina cf. *hansbechi* Schmeckel and Cappellato, 2001		Mataró, Spain	MNCN 15.05/88108	MK32300*	MK322973*	MK323028*
Runcina ferruginea Kress, 1977		Newlyn, Cornwall, England	MNCN 15.05/88109	MK323011*	MK322977*	MK323032*
Runcina hornae Schmeckel and Cappellato, 2002		Palamós, Spain	MNCN 15.05/88110	-	MK322978*	MK323033*
Aplysia dactylomela Rang, 1828		Cape Verde (GB)	NHMUK 20030795/ 20030796	KJ022921	KF992168	KJ022798
Micromelo undatus (Bruguière, 1792)		Tenerife, Canary Island (GB)	NHMUK 20030800	KJ022944	DQ974653	KJ022778

Table 3. Differences between *Runcina brenkoae*, *Runcina lusitanica* sp. nov. and *Runcina marcosi* sp. nov. Data after Thompson (1980), Thompson and Brodie (1988), Schmekel and Cappellato (2002) and present study.

	Runcina brenkoae	*Runcina lusitanica* sp. nov.	*Runcina marcosi* sp. nov.
Colour pattern	Body red-brown, sometimes translucent pale fawn. Anastomosing dark blotches on notum, margin, and sole of foot. Chalk white spots all over body, more concentrated on margin of tail, both sides of head behind eyes and on metapodium in front of the dark band. Longitudinal band of dark brown or wine red on the surface of the metapodium.	Body brown and translucent yellowish. Anastomosing dark blotches on notum and margin of foot. Some specimens present a large pale fawn patch on posterior part of head and notum. Longitudinal band, sometimes wide, of dark brown on surface of metapodium.	Body red-brown or translucent pale fawn. Anastomosing dark or reddish blotches on notum, margin of foot and metapodium. White spots all over the body. Longitudinal band of dark brown or wine red on the surface of metapodium.
Gills	Two equal-sized translucent gills with white spots bearing pinnules.	Two big yellowish gills with dark spots bearing irregular pinnules. Upper gill unipinnate and ventral bipinnate.	Two translucent gills. Upper unipinnate and ventral bipinnate.
Shell	Absent	Absent	Absent
Radular formulae	20 x 1.1.1	25–29 x 1.1.1	10–13 x 1.1.1
Radula teeth	Rachidian tooth bilobed. Two pads cockle-shaped with 8–10 denticles each. Lateral teeth smooth, elongate and curved like a swan neck.	Rachidian tooth bilobed. Two flat pads with 10–12 denticles each. Lateral teeth smooth, elongate and curved like a swan neck.	Rachidian tooth bilobed. Two flat pads comb shaped with 10–11 denticles each. Lateral teeth smooth, elongate and curved like a swan neck.
Gizzard plate	Four plates with 5–6 crests	Four plates with 10–11 crests	Four plates with 7–8 crests

SUPPLEMENTARY FIGURES

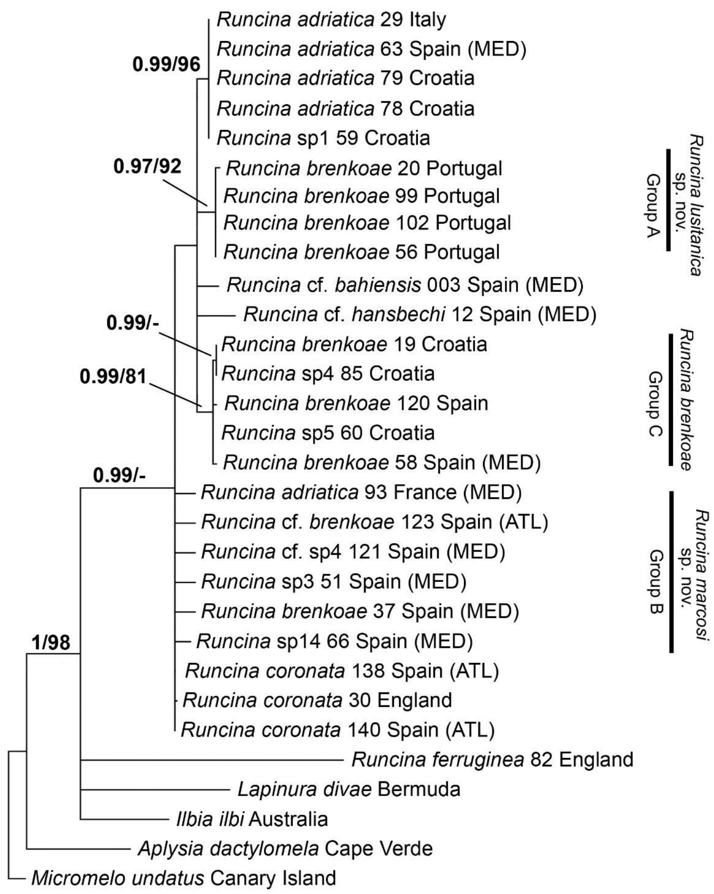

Figure S1. Phylogenetic hypothesis based on BI of the H3 gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

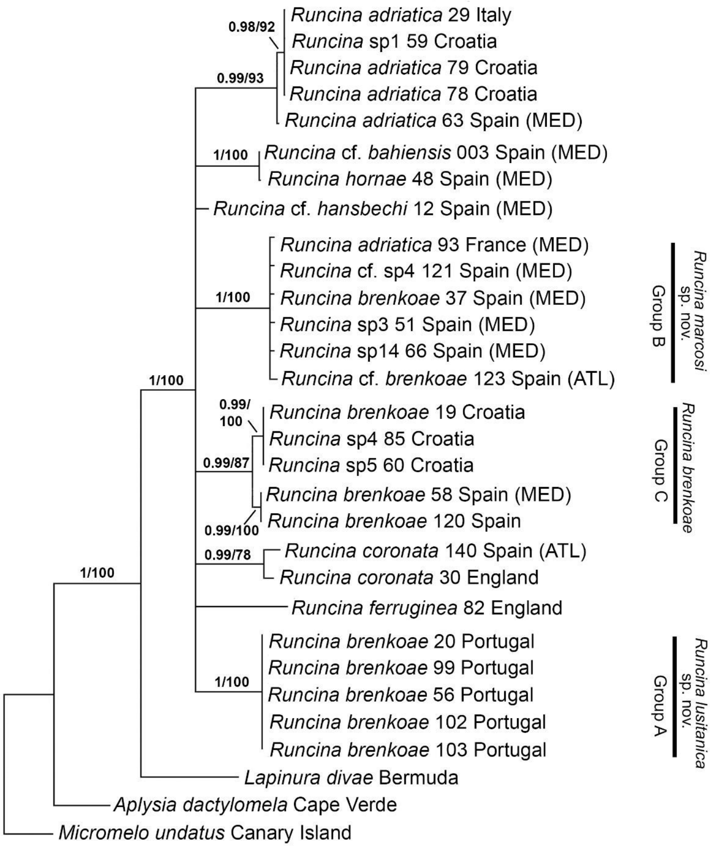

Figure S2. Phylogenetic hypothesis based on BI of the COI gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

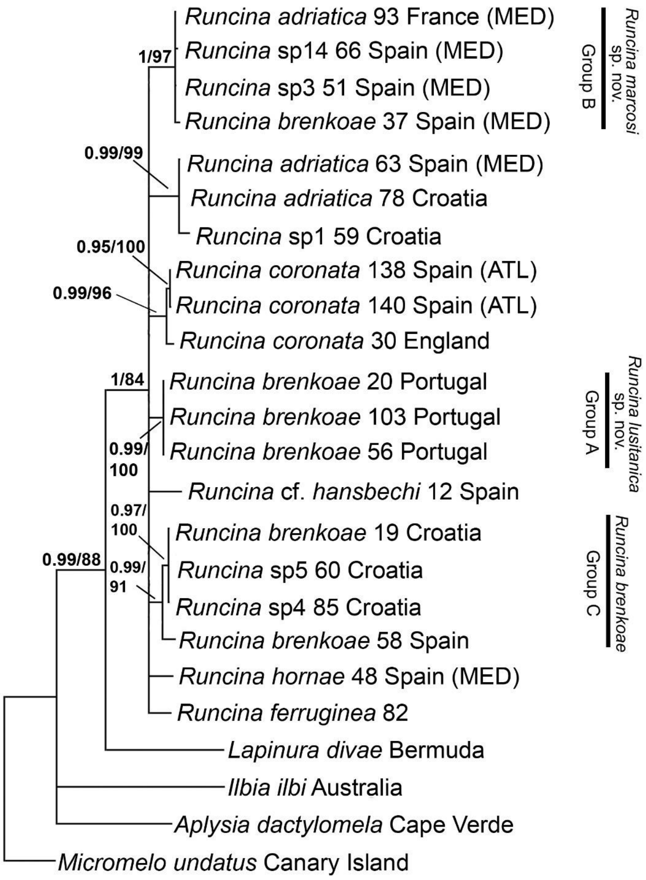

Figure S3. Phylogenetic hypothesis based on BI of the 16S gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

Capítulo 3

Integrative taxonomy reveals that not all European reddish runcinids are *Runcina ferruginea* Kress, 1977 (Gastroposa, Heterobranchia)

Declaración: Esta obra no se considera publicada a los efectos previstos en el Art. 8.2 del Código Internacional de Nomenclatura Zoológica. No constituye, por tanto, un registro científico público y permanente para fines de nomenclatura zoológica.

Integrative taxonomy reveals that not all European reddish runcinids are *Runcina ferruginea* Kress, 1977 (Gastroposa, Heterobranchia).

ABSTRACT

Runcina ferruginea Kress, 1977 is a reddish-brown runcinid described from Plymouth, southwestern of England. Over the years, several reddish runcinids has been identified as *R. ferruginea* from Portugal to Mediterranean coast of France. Due to some similarities between *R. ferruginea* and the Croatian species *R. zavodniki* described by Thompson, 1980, the latter has been considered as junior synonym of the former. The results presented in Chapter I revealed *Runcina ferruginea* as a complex species including four species. Therefore, in this chapter we investigate the taxonomic status of the species included in this complex through an integrative approach, combining multi-locus molecular phylogenetics, species delimitation analyses (ABGD, bPTP) and morpho-anatomical characters, based on specimens from southwestern of England, central and western Mediterranean Sea (Spain, France, Italy and Croatia). Herein, we redescribe *R. ferruginea* proper and confirm *R. zavodniki* as a valid species. Two new species are described, one belonging to the genus *Runcina* (*R. lupiaensis* sp. nov.) and another species belonging to a different genus, here proposed as *Pseudoruncina* gen. nov. (*Pseurorucina marinae* sp. nov.).

INTRODUCTION

Within the Runcinida, only the species *Runcina coronata* (Quatefrages, 1844), *Runcina capreensis* Mazzarelli, 1893, *Runcinella zelandica* Odhner, 1924, *Metaruncina setoensis* (Baba, 1954), *Lapinura divae* (Ev. Marcus and Er. Marcus, 1963) and *Runcina ferruginea* Kress, 1977 have been morpho-anatomically well studied (Vayssière, 1883, Mazzarelli, 1893, Odhner, 1924, Ghiselin, 1963, Baba, 1967, Er. Marcus and Ev. Marcus, 1970, Kress, 1977). Among them, *Runcina ferruginea* is characterized by having a homogeneously reddish-brown coloured body (notum, foot and gills) with very fine dark brown spots all over the body as well as a detailed description of the digestive and reproductive system based on histological serial sections (Kress, *op. cit*., 1985a, b, 1986, Kress and Schmekel, 1992, Kress *et al*., 1994). Moreover, *R. ferruginea* is characterized by an elongated body, head with two small lobes in the front, three small pinnate gills right to the anus and shell absent. The digestive gland is visible through the skin as an oval dark brown blotch. Due to its very simple colour pattern, *R. ferruginea* could be easily confused with two others "red *Runcina* species" described by Ortea and Moro (1999) from Tenerife, Canary Islands, *Runcina hidalgoensis* Ortea and Moro, 1999 and *Runcina medanensis* Ortea and Moro, 1999, which they called "*ferruginea* group".

Runcina ferruginea was originally described from Plymouth, southwestern of England, but it has also been recorded in Portugal (Ortea and Moro, 1999), Spain (Ortea and Urgorri, 1981 [Galicia, northwestern coast], Cervera, García-Gómez and García, 1991 [southern Andalusia], Ballesteros *et al*., 2016 [Catalonia, northeastern coast]), France [Mediterranean coast] and Croatia (Schmekel and Cappellato, 2002). However, reddish runcinids from Croatia were described by Thompson (1980) under the name *R. zavodniki*. In the original description of this species, most specimens were characterized by having an elongated body, three pinnate gills and a black colour (including foot and gills), except for one specimen with pale orange-brown colour. Later, Thompson and Brodie (1988) referred to more specimens of *R. zavodniki* collected near to the type locality, Rovinj (Croatia). These specimens showed black, red-brown and pale orange-brown colour and significant difference at the radula with the lateral teeth smooth while in the original description these teeth showed several fine denticles, which the authors considered to be a mistaken observation. Due to some similarities between *R. ferruginea* and *R. zavodniki* [sensu Thompson and Brodie (1988)] (body shape, radula, gills), Schmekel and Cappellato (2002) considered these two species to be conspecific and *R. zavodniki* was considered as junior synonym of *R. ferruginea*. Prkić *et al*. (2018) reinstated the name *R. zavodniki*, but without further explanation.

Several complexes of cryptic species among heterobranch sea slugs have been discovered with the use of integrative taxonomic approaches and in particular of molecular phylogenetics (Padula *et al*., 2014, Carmona *et al*., 2015, Krug *et al*., 2018, Valdés *et al*., 2018). Recently, Araujo *et al*. (2019) (see Chapter II) showed that the variable chromatic patterns described for *Runcina brenkoae* Thompson, 1980 hide a complex of species among runcinids. In Chapter I, our phylogenetic analyses revelead *R. ferruginea* as another species complex within the order Runcinida. In this contribution, we investigate the taxonomic status of the species *Runcina ferruginea* and also reevaluate the status of *R. ferruginea* and *Runcina zavodniki* through an integrative approach that combines multi-locus molecular phylogenetics and morpho-anatomical characters, based on specimens from the southwest of England, central and western Mediterranean Sea (Spain, France, Italy and Croatia).

MATERIAL AND METHODS

DNA extraction, amplification and sequencing

All specimens used in this study were those obtained in Chapter I and from Genbank (Table 1). *Micromelo undatus* (Bruguière, 1792) was used to root the tree as outgroup taxon. Tissue samples were taken from the foot and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). DNA extraction, amplification and sequencing follow Chapter I of this Thesis.

The type specimens of *Runcina ferruginea* were originally preserved in formalin 5% and thus, DNA sequencing was not viable. Specimens from the type locality, Plymouth (sourthwestern England) could not be obtained, but we sequenced one specimen from nearby locality, Newlyn, 104 km away to west from Plymouth.

Phylogenetic analyses

Sequences were edited in Geneious v10.2.3 (Drummond *et al.* 2009) and aligned using MAFFT (Katoh *et al.*, 2009) implemented in Geneious v10.2.3 (Drummond *et al.* 2009) with the default settings (Auto [FFT-NS-1, FFT-NS-2, FFT-NS-i or L-INS-i; depends on data size]). Sequences from the protein-coding genes COI and H3 were translated into amino acids to check for stop-codons. Hypervariable regions of the 16S alignment where homology could not be confidently established were removed using Gblocks under relaxed settings (Talavera and Castresana, 2007). Nevertheless, analyses including and excluding these regions provided similar results. Therefore, final analyses were performed including all bases. Sequences of the COI, 16S and H3 genes were trimmed to 658, 461 and 328 nucleotides, respectively. All three genes were concatenated using Mesquite v3.2 (Maddison and Maddison, 2018), resulting in a final dataset of 1447 base pairs. Single gene and a concatenated (H3+COI+16S) analyses were performed. Saturation for the first, second and third codon positions of the COI and H3 genes were calculated in MEGA v7.0 (Kumar *et al.*, 2016).

The best-fit evolutionary model for each gene was determined in jModeltest v2.1.6 (Guindon and Gascuel, 2003; Darriba *et al.*, 2012), under the Akaike information criterion (AIC) (Akaike, 1974). The GTR + G + I model was selected for all three genes. Bayesian inference (BI) analyses were performed in MrBayes v. 3.2.1 (Ronquist and Huelsenbeck, 2003) with a random starting tree and two parallel runs of 10^7 generations. Convergence was checked in TRACER v1.7.1 (Rambaut *et al.*, 2018) with a burn-in of 25%. Nodes with a posterior probability (PP) ≥ 0.95 (Alfaro, Zoller and Lutzoni, 2003) were considered well supported and discussed. Maximum likelihood (ML) analysis was executed using RAxML v8 (Stamatakis, 2014) and node support was assessed with nonparametric bootstrapping (BS) with 5000 replicates. Nodes with bootstrap values (BS) ≥ 70 (Hillis and Bull, 1993) were considered significant and were discussed. Both BI and ML trees were visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Minimum and maximum pairwise uncorrected *p*-distances of COI within and between species were calculated in MEGA v7.0 using all sequences available (Kumar *et al*., 2016).

Species delimitation analyses

The Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al*., 2012) and the Bayesian Poisson tree processes (bPTP) (Zhang *et al*., 2013) analyses were used to aid delimiting species. For the ABGD we used the alignment from the fast-evolving COI gene with default settings ($P_{\text{min}} = 0.001$, $P_{\text{max}} = 0.1$, Steps = 10, $X = 1.2$, Nb bins = 20), under the three models of evolution available, namely Jukes-Cantor (JC69), Kimura (K80) and Simple Distance. The bPTP analysis is an updated version of the original maximum likelihood PTP (modelling speciation in terms of the number of substitutions), which adds Bayesian support values to delimit species. The bPTP analyses were run with the COI and 16S trees using the webserver (https://species.h-its.org/ptp/) (Zhang *et al*., 2013).

Morphology

To complete and compare the results obtained by molecular phylogenetics and species delimitation analyses, reddish/brownish specimens previously identified as *Runcina ferruginea* from Newlyn (England) (1), Catalonia (northeastern Spain, Mediterranean Sea) (1), Lecce (southern Italy) (5) Croatia (Adriatic) (3), and Banyuls-sur-Mer (France, Mediterranean Sea) (1) were studied anatomically. Animals were dorsally/ventrally dissected and the buccal bulbs were extracted and dissolved in a solution of 10% sodium hydroxide to expose the radula. The radulae and gizzard plates were then immersed in water, dried and mounted for scanning electron microscopy (SEM) with a Nova NanoSEM 450 available at the University of Cadiz (Cadiz, Spain). The reproductive systems were examined and drawn using a dissecting microscope with the aid of a *camera lucida*.

RESULTS

Phylogenetic analyses

Saturation was not observed, not even in the third codon position. The combined ML and BI trees (H3+COI+16S, Fig. 1) provided better resolution than the individual gene analyses (Figs S1, S2 and S3). The species *Ilbia ilbi* was rendered sister to Runcinidae (Clade I; PP = 0.99; BS = 97). Clade I split into two clades but the relationship between Clade II and Clade III was only recovered by the BI analysis (PP = 0.98). Within Clade II, a sister relationship between

Lapinura divae and the subclade with specimens of *R. ferruginea* from Mediterranean Spain and France coasts was also only recovered by the BI analysis (PP=0.98). Regarding Clade III, *Metaruncina setoensis* resulted to be sister lineage of the genus *Runcina* (orange square) (PP = 1; BS = 88). In Both BI and ML analyses, the monophyly of *Runcina* was supported only when the specimens of *R. ferruginea* included in Clade II (green square) were excluded (PP = 1; BS = 92).

The specimens previously identified in this study as *R. ferruginea* split into four clades, three of them with maximum support (PP = 1; BS = 100). The first clade (Group RF1) clustered outside of the *Runcina* clade (orange square), with specimens from northeastern Spain (Catalonia, Mediterranean Sea) and France (Banyuls-sur-Mer, Mediterranean Sea); the second clade (Group RF2) included specimens from southern Italy; the third clade (Group RF3) contained specimens from Croatia (Adriatic Sea) and the fourth clade (Group RF4) with only one specimen from England (Fig. 1).

Species delimitation analyses

The ABGD analysis of the COI sequences with all three models of evolution resulted in 17 groups with four of them (groups RF1, RF2, RF3 and RF4) corresponding to the same *R. ferruginea* clades recovered by the BI and ML analyses (Fig 1A). The results obtained with the bPTP analysis were congruent with the ABGD output, by suggesting the same four groups of *Runcina ferruginea* (Fig. 1B).

Regarding the COI uncorrected *p*-distances (Table 2), the minimum distance among all four groups ranged from 9% to 18.3%. The maximum distance within specimens of Group RF1 was 0.2%, within Group RF2 1.9%, and within Group RF3 was 1.5%. The Group RF4 had one single specimen. The COI uncorrected *p*-distances between species in the genus *Runcina* ranged from 0.0% to 14.9%, while among species of different genera of Runcinida ranged from 15% to 21% (Table 3). No COI gene sequences from *Ilbia ilbi* were available for this analysis.

Our molecular results support the occurrence of four species under the name *Runcina ferruginea*. Based on our phylogenetic tree, the species delimitation analyses and the pairwise uncorrected distance, it is clear that three of these species actually belong to the genus *Runcina*. However, the specimens included in Group RF1 from northeastern Spain and France, both in Western Mediterranean Sea from localities close to each other, seem to belong to a different genus, as they constitute a monophyletic and highly supported group (PP = 1; BS =100). These results are backed by morphological differences across specimens from the four "*R. ferruginea*" clades and are formally described and/or redescribed in the Systematic section below.

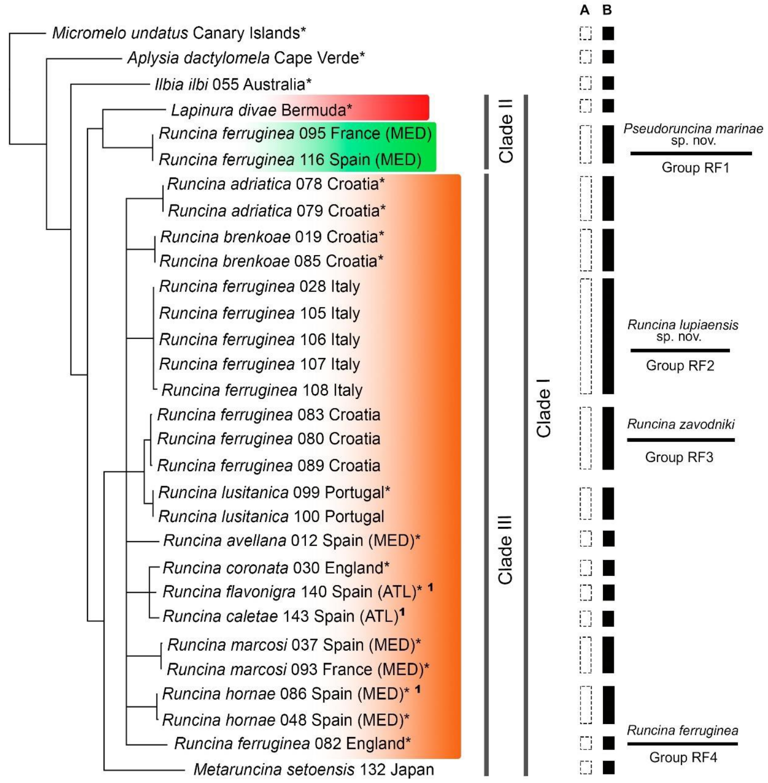

Figure 1. Phylogenetic hypothesis of "*Runcina ferruginea*" complex based on the combined dataset (H3+COI+16S) inferred by Bayesian Inference. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from Maximum Likelihood. (**A**) ABGD results based on the COI data set. (**B**) bPTP results based on the COI data set. Abbreviations: ATL, Atlantic Ocean; MED, Mediterranean Sea. (*) Refers to sequences from Genbank. (1) Refers to names proposed in Chapter I.

Table 2. Uncorrected *p*-distances based on COI sequences from specimens identified as *Runcina ferruginea*.

	Distance between groups (%)			Distance within groups (%)
	Group RF1	Group RF2	Group RF3	
Group RF1				0.2
Group RF2	16.4–17.2			0.0–1.9
Group RF3	16.6–17	9.0–10.5		0.3–1.5
Group RF4	18.3–18.5	9.8–11.1	9.6–10.4	-

Table 3. Uncorrected *p*-distances (%) based on COI sequences among genera species of different genera of Runcinida

	Runcina	***Lapinura***	***Metaruncina***
Runcina	0.0–14.9		
Lapinura	15–20.4		
Metaruncina	15.8–20.1	19.5	
***Pseudoruncina* gen. nov.**	16.3–18.5	17.7–17.8	20.8–21

Systematic description

Family RUNCINIDAE H. Adams and A. Adams, 1854
Runcina Forbes in Forbes and Hanley, 1851

Runcina ferruginea Kress, 1977
(Figs 2A, B; 3A–C; 4A; 5A)

Runcina ferruginea Kress, 1977: figs 1–4, Pl. Ia, c–e, Pl. IIc–g; Ortea and Urgorri, 1981: 149–150, fig. 1A; Fernandez-Ovies, 1983: plates I–IV; Thompson and Brodie, 1988: 345, fig. 1f; Cervera, García-Gómez and García, 1991: 202–203, fig. 4; Ortea and Moro, 1999: 64, fig. 1, plate 1A; Schmekel and Capellato, 2001: 144–145, Pl. If, Pl. IIn; Schmekel and Capellato, 2002: 97–99, Pl. Ia, IVd–h; Ballesteros *et al*., 2016: 4, fig. 7b.

Type material: Holotype Museum of Natural History, Basel, Switzerland (not studied because the material is fixed in formalin 5%).

Type locality: Plymouth, England.

Distribution: England ([southern] Kress, 1977, present study); Portugal (García Gómez *et al*., 1991, Calado *et al*., 1999, Ortea and Moro, 1999); Spain (Ortea and Urgorri, 1981 [Galicia, northwestern coast], García Gómez *et al*., 1989 [Ceuta, Strait of Gibraltar, North Africa], Cervera, García-Gómez and García, 1991 [Strait of Gibraltar, southern Andalusia], Ballesteros *et al*., 2016 [Catalonia, northeastern coast]); France [Mediterranean coast] (Schmekel and Cappellato, 2002).

Examined material: MNCN 15.05/88109, Newlyn, England, 26 Mar 2018, 3.5 mm in length, David Fenwick coll. (dissected and sequenced).

External morphology (Fig. 2A, B): Elongated body with homogeneous reddish colour. Small dark spots all over the body, visible under stereoscopic microscope and also in preserved animal. Fine dark line on the margin of the notum and foot. Notum smooth as wide as foot. Head zone shallowly bilobed in front (Fig. 2B). Posterior end of the notum rounded. Foot rounded at both ends extending posteriorly beyond the notum. Anteriorly extending laterally on the head zone. Oral tentacles absent. Eyes and digestive gland (dark blotch) visible through the skin. Three reddish pinnate gills on the right side of the anus. Anus situated on the median line of body, beneath the notum. Shell absent.

Internal anatomy (Figs 3A–C, 4A, 5A): Radular formula 20 x 1.1.1 (MNCN 15.05/88109). Rachidian tooth boomerang shaped with smooth lateral wings on each side. Central part of rachidian tooth bilobed; masticatory edge contains a pair of pads curved on the top; each pad possesses 15 long laminae-shaped denticles, and one inner and outer undeveloped denticle. Median deep and broad depression present between the pads (Fig. 3A). Lateral teeth smooth, elongate and hooked-shape. Apical cusp strongly curved. Inner part of the basal region pronounced (Fig. 3B). Triangular jaws present. Four gizzard plates with 7–8 crests (Fig. 3C). Reproductive system monoaulic. Female gland mass divided into three lobes. Long and relatively wide common genital duct connecting the female gland to the exterior on the posterior right side of the body (Fig. 4A). Female gland placed beneath the posterior right side of digestive gland. Male copulatory organ opens to the right of the mouth. Unarmed, rounded penial papilla projects into a short atrium. Prostate gland long, cylindrical, broad and curved. Oval seminal vesicle narrowly connected to prostate. Almost half size of prostate gland (Fig. 5A).

Runcina zavodniki Thompson, 1980

(Figs 2C, D; 3D–F; 4B; 5B)

Runcina zavodniki Thompson, 1980: 156, fig. 1d, Table 1; Thompson and Brodie, 1988: 342–343, fig. 1c; Prkić *et al*. 2018: 123, figs 4–5.

Type material: Holotype (NHMUK 197914W) Natural History Museum, London, UK (not studied because the material is only available as micro-slide preparations).

Type locality: Rovinj, Croatia.

Distribution: Croatia (Thompson, 1980, Thompson and Brodie, 1988, Prkić *et al*., 2018 and present study).

Examined material: MNCN 15.05/90669, Split, Croatia, Jakov Prkić coll., 08 Sep 2013, 3 mm in length, depth 1m, found washing *Posidonia* (dissected and sequenced). MNCN 15.05/90667, Iž Island, Croatia, Alen Petani coll., 13 Aug 2017, 2 mm in length, depth 7–16m (dissected and sequenced). MNCN 15.05/90668, Ugljan Island, Croatia, Alen Petani coll., 06 Sep 2017, 2 mm in length, depth 1–3m (dissected and sequenced).

External morphology (Fig. 2C, D): Body elongated with colour ranging from dark to orange or brownish. Fine dark line on the margin of the notum and foot. Notum smooth and as wide as the foot. Head straight in front. Posterior part of the notum rounded. Anterior part of the foot is rounded and wider than the notum. Foot rounded posteriorly and extending beyond the notum end. In clearer specimens, eyes and digestive gland (dark blotch) visible through the skin. Three pinnate gills right to the anus. Gills with the same colour of the body. Anus situated in median line of body, beneath the notum. Shell absent.

Internal anatomy (Figs 3D–F; 4B; 5B): Radular formulae 20 x 1.1.1 (MNCN 15.05/90669), 24 x 1.1.1 (MNCN 15.05/90668) and 29 x 1.1.1 (MNCN 15.05/90667). Rachidian tooth boomerang shaped with smooth lateral wings on each side. Central part of rachidian tooth bilobed; masticatory edge contains a pair of flattened pads; each possessing 8–10 long denticles

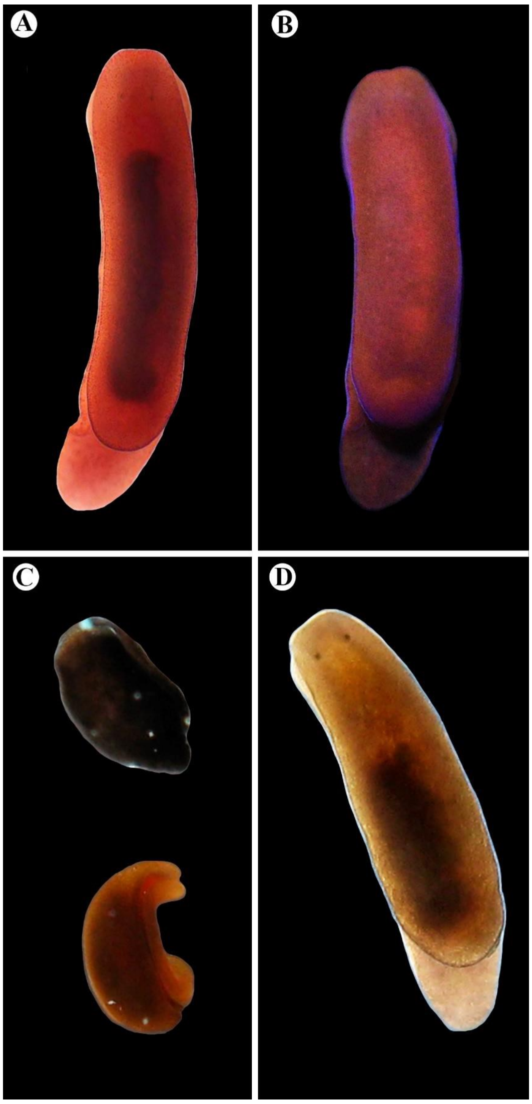

Figure 2. Living animals of *Runcina ferruginea* **(A–B)** and *R. zavodniki* **(C–D)**. **A, B.** MNCN 15.05/88109 specimen from Plymouth, England (photos David Fenwick); **C.** MNCN 15.05/90667 specimen from Iž Island, Croatia (Photo Alen Petani); **D.** MNCN 15.05/90669 specimen from Split, Croatia (Photo Jakov Prkić).

laminae-shaped disposed as an arc. Denticles with same size and sharp tip. Median broad depression present between the pads; one to three very small denticles arranged in layers present in this depression (Fig. 3D). Lateral teeth triangular with curved tweezers shape bearing about 20 smalls, pointed denticles (Fig. 3E). Triangular jaws present. Four gizzard plates with 6 – 8 crests (Fig. 3F). Reproductive system monoaulic. Small female gland mass slightly divided into three lobes. Long, curved and broad common genital duct opens to the exterior on the posterior right side of the body (Fig. 4B). Female gland placed beneath the posterior right side of digestive gland. Male copulatory organ opens to the right of the mouth. Penial papilla not observed. Prostate gland long, broadly rounded on the posterior part and slightly narrow towards the aperture. Seminal vesicle curved, cylindrical and relatively large, almost half size of the prostate. Connected to prostate gland at the second third of its length (Fig. 5B).

Runcina lupiaensis **sp. nov.**

(Figs 3G–I; 4C; 5C; 6A)

Distribution: Lecce, southeastern Italy (present study).

Examined material: Holotype: MNCN (pending number of voucher), St. Andrea Island, Gallipoli Lecce (Italy), coll. Fabio Vitale, 02 Jun 2018, 1.2 mm in length preserved (sequenced). Paratype: MNCN (pending number of voucher), Otranto, Lecce (Italy), coll. Fabio Vitale, 30 Aug 2015, 2 mm in length preserved (dissected and sequenced). Additional materials: MNCN (pending number of voucher), St. Andrea Island, Gallipoli, Lecce (Italy), coll. Fabio Vitale, 02 Jun 2018, 1 mm in length preserved (dissected and sequenced). MNCN (pending number of voucher), St. Andrea Island, Gallipoli Lecce (Italy), coll. Fabio Vitale, 02 Jun 2018, 0.75 mm in length preserved (dissected and sequenced). MNCN (pending number of voucher), St. Andrea Island, Gallipoli Lecce (Italy), coll. Fabio Vitale, 02 Jun 2018, 1 mm in length preserved (sequenced).

Etymology: The Latin word *lupiaensis* refers to the Latin name (Lupiae) of the Italian Province of Lecce.

External morphology (Fig. 6A): Body elongated with homogeneous reddish-brown colour. Fine dark line on the margin of the notum and foot. Dark small spots absent. Notum smooth and slightly wider than foot. No lobes on the head zone. Head straight in front. Notum broadly rounded on the posterior end and slightly narrow towards the head. Anterior part of the foot is

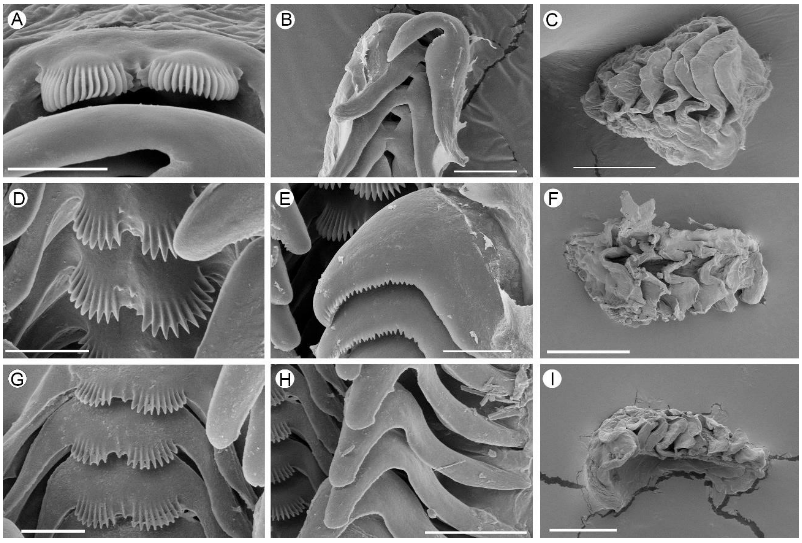

Figure 3. Scanning electron micrographs of radula and gizzard plates of *Runcina* species. **A, B, C**. *R. ferruginea* (MNCN 15.05/88109). **A**. Rachidian radular tooth. **B**. Lateral radular teeth. **C**. Gizzard plate. **D, E, F**. *R. zavodniki*. (MNCN 15.05/90667, MNCN 15.05/90669) **D**. Rachidian radular teeth (MNCN 15.05/90667). **E.** Lateral radular teeth (MNCN 15.05/90667). **F**. Gizzard plate (MNCN 15.05/90669). **G, H, I**. *R. lupiaensis* sp. nov. (MNCN pending number of voucher). **G**. Rachidian radular teeth (MNCN pending number of voucher). **H**. Lateral radular teeth (MNCN pending number of voucher). **I**. Gizzard plate (MNCN pending number of voucher). Scale bars: **A** = 4 µm; **B, H** = 10 µm; **C** = 40 µm; **D, E, G** = 5 µm; **F, I** = 50 µm;

rounded and wider than the notum. Foot rounded posteriorly and extending beyond the notum end. Eyes difficult to discern. Digestive gland sometimes visible as dark blotch. Three reddish-brown pinnate gills right to the anus. Anus situated in median line of body, beneath the notum. Shell absent.

Internal anatomy (Figs 3G–I; 4C; 5C): Radular formulae 23 x 1.1.1 (MNCN pending number of voucher) and 27 x 1.1.1 (MNCN pending number of voucher). Rachidian tooth boomerang shaped with long and smooth lateral wings on each side. Central part of rachidian tooth bilobed; masticatory edge contains a pair of flat and short pads, each one possessing 7–10 long, thin laminae-shaped denticles with relatively the same size. Denticles with sharp tips. Median broad depression present between the pads; one small denticle present; in some rows two denticles arranged in layers are present (Fig. 3G). Lateral teeth smooth, elongate and swan neck shaped (Fig. 3H). Triangular jaws present. Four gizzard plates with 10 crests (Fig. 3I). Reproductive system monoaulic. Large female gland mass without visible lobes. Bursa copulatrix absent. Common genital duct opens to the exterior on the posterior right side of the body. Genital duct relatively long and narrow, becoming broad at the connection zone with the female mass (Fig. 4C). Female gland placed beneath the posterior right side of digestive. Male copulatory organ opens to the right of the mouth. Long unarmed penial papilla projects into the atrium. Prostate gland long, cylindrical and strongly curved. Seminal vesicle elongated, slender and curved reaching half size of prostate gland (Fig. 5C).

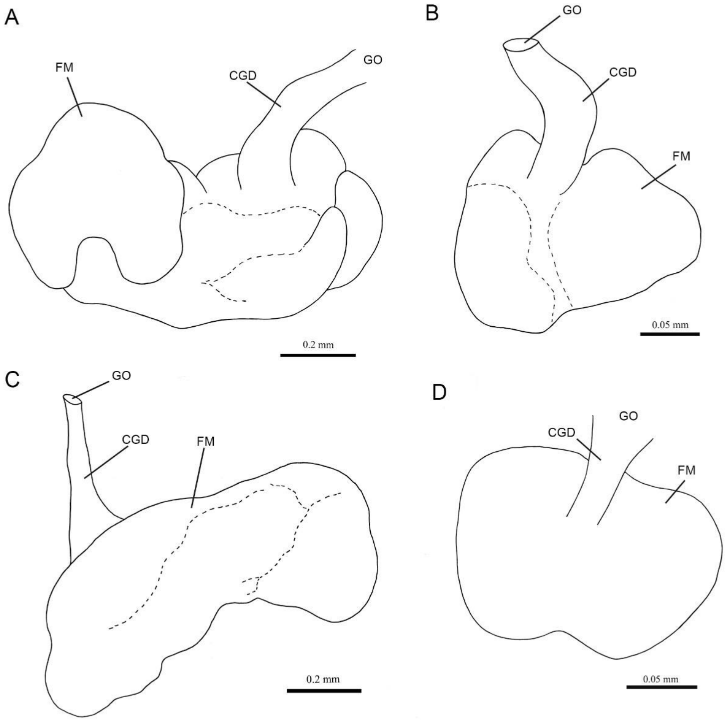

Figure 4. Female reproductive system of *Runcina* species. **A**. *R. ferruginea*, Newlyn, England (MNCN 15.05/88109). **B**. *R. zavodniki* Iž Island, Croatia (MNCN 15.05/90668). **C**. *R. lupiaensis* sp. nov. Otranto, Italy (MNCN pending number of voucher). **D.** *Pseudoruncina marinae* sp. nov. Roses, Spain (Mediterranean Sea) (MNCN pending number of voucher). Abbreviations: FM, female mass; CGD, common genital duct; GO, gonopore.

Pseudoruncina gen. nov.

Diagnosis: A narrow elongated runcinid with three small gills. Jaw present. Radula absent. Shell absent.

Distribution: To date, only known from Roses (northeastern Spain) to Banyuls-sur-Mer (Mediterranean coast of France).

Etymology: the name refers to the external similarity respect to the species of the genus *Runcina*.

Type specie: *Pseudoruncina marinae* sp. nov.

Pseudoruncina marinae sp. nov.

(Figs 4D; 5D; 6B, C; 7)

Distribution: To date, only known from Roses (northeastern Spain) to Banyuls-sur-Mer (Mediterranean coast of France).

Examined material: Holotype: MNCN (pending number of voucher), Roses, Catalonia, northeastern, Spain, coll. Marina Poddubetskaia, 09 July 2018, 1 mm in length, depth 11m (dissected and sequenced). Paratype: ZSM MOL 201442074, Banyuls-sur-Mer, France (Mediterranean), coll. Bastian Brenzinger and Timea Neusser, 02 July 2014, 1 mm in length preserved (sequenced).

Etymology: This species is dedicated to Marina Poddubestkaia who kindly provided us specimens of several Runcinida species from Spain and France.

External morphology (Fig. 6B, C): Body elongated. Reddish colour covering the notum and anterior margins of the foot. Fine dark line on the margin of the notum and foot. Mantle furrow, median and posterior part of the foot with translucent colour. Notum smooth and slightly wider than the foot. Anterior and posterior part of the notum rounded. Foot anteriorly rounded; posteriorly short, half-hexagon-shaped extending beyond the notum. Small dark spots dispersed beneath the notum, on the mantle furrow and on the ventral margin of the surface of the foot. Eyes visible laterally on the mantle furrow and difficult to observed from above on the head zone. Digestive gland visible through the skin. Three small pinnate gills right to the anus. Gills light red colour. Anus situated in median line of body, beneath the notum. Shell absent.

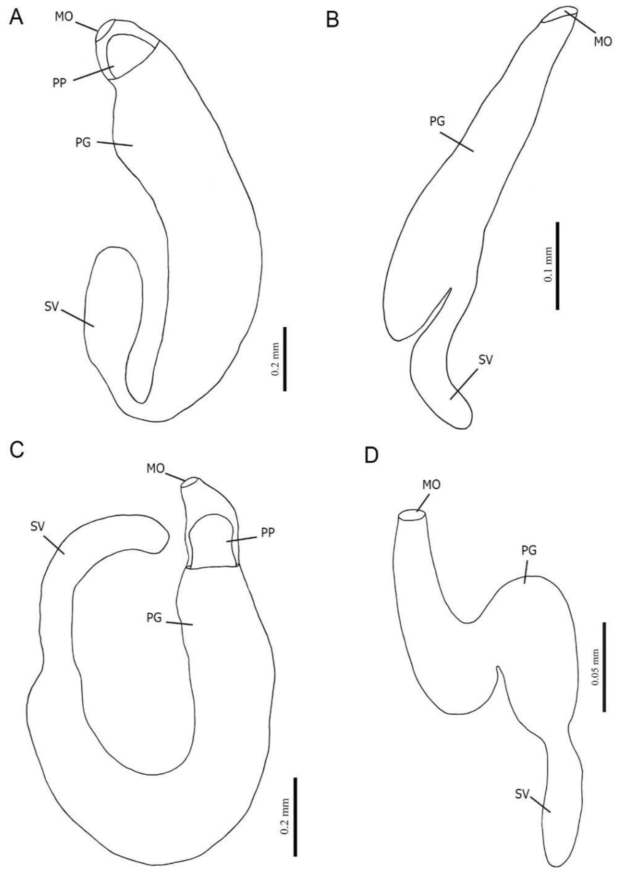

Figure 5. Male reproductive system of *Runcina* species. **A**. *R. ferruginea*, Newlyn, England (MNCN 15.05/88109). **B**. *R. zavodniki* Iž Island, Croatia (MNCN 15.05/90668). **C**. *R. lupiaensis* sp. nov. Otranto, Italy (MNCN pending number of voucher). **D.** *Pseudoruncina marinae* sp. nov. Roses, Spain (Mediterranean Sea) (MNCN pending number of voucher). Abbreviations: MO, male opening; PP, penial papilla; PG, prostate gland; SV, seminal vesicle.

Internal anatomy (Figs 4D; 5D; 7): Radula absent. Triangular jaws present. Four gizzard plates with 7 lamellae (Fig. 7). Reproductive system monoaulic. Female gland mass almost rounded, apple-shaped. Bursa copulatrix absent. Common genital duct short, narrower in length and broader at the outer opening (Fig. 4D). Common genital duct opens to the exterior on the posterior right side of the body. Female gland placed beneath the posterior right side of digestive gland. Male copulatory organ opens to the right of the mouth. Penial papilla not observed. Prostate gland cylindrical and strongly curved, in shape of an "S" lying down. Thick pickles-shaped seminal vesicle relatively large, almost half size of the prostate gland (Fig. 5D).

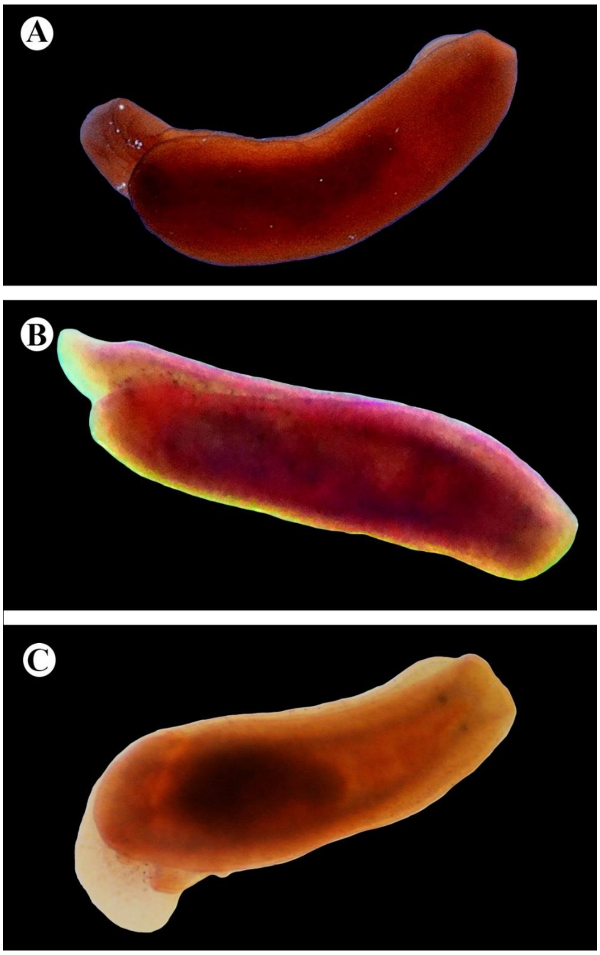

Figure 6. Living animals of *Runcina lupiaensis* sp. nov. **(A)** and *Pseudoruncina marinae* sp. nov. **(B–C)**. **A.** (MNCN pending number of voucher) specimen from Otranto, Italy (photo Fabio Vitale); **B.** (MNCN pending number of voucher) specimen from Roses, Spain (Mediterranean Sea) (photo Marina Poddubetskaia); **C.** (MNCN pending number of voucher) specimen from Banyuls-sur-Mer, France (Mediterranean Sea) (photo Bastian Brenzinger).

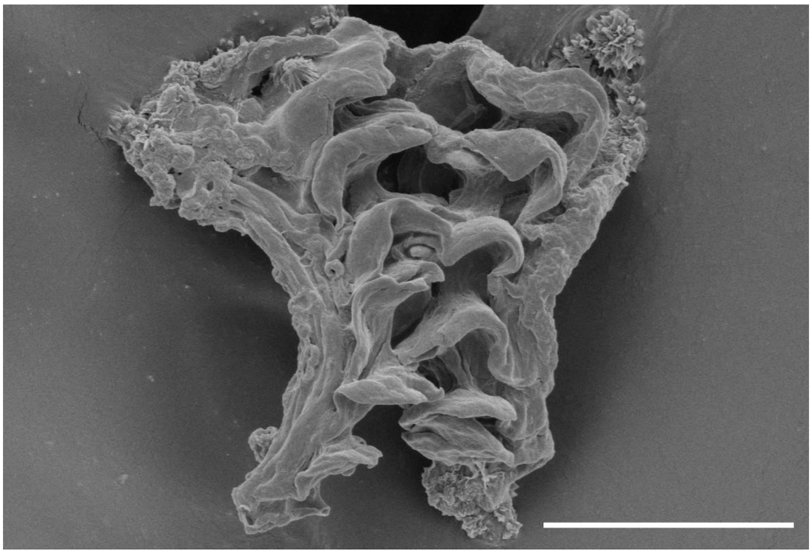

Figure 7. Scanning electron micrographs of gizzard plates of *Pseudoruncina marinae* sp. nov. (MNCN pending number of voucher). Scale bar: 40 µm.

DISCUSSION

R. ferruginea has always been an easily recognizable species by its simple and marked reddish colour pattern. However, our phylogenetic analyses have revealed a new species complex within "*R. ferruginea"*, recognizing three distinct species within the genus *Runcina*: *R. ferruginea*, *R. zavodniki* and *R. lupiaensis* sp. nov. plus, a new species belonging to a distinct genus, here proposed as *Pseudoruncina* gen. nov.

Specimens of *R. ferruginea* from northeastern Spain and Mediterranean coast of France were preliminary ascribed to *Runcina* due to their external morphology similarity with the species of this genus. In our study, these specimens resulted to be the same species (uncorrected *p*-distances = 0.0–0.2% between specimens) constituting a monophyletic and high supported clade (Fig. 1, green square). This clade is sister to *Lapinura divae*, but should not be included with *Lapinura* due to anatomical differences like the shape of the body, presence of radula and shell, for example. Therefore, the specimens from Mediterranean coast of Spain and France early identified as *Runcina ferruginea* were grouped in the new proposed genus *Pseudoruncina* gen. nov.

Thompson (1980) described *Runcina zavodniki* with lateral teeth denticulate. However, in a posterior complementary description, Thompson and Brodie (1988) observed that in additional collected specimens of *R. zavodniki* from type locality (Rovinj, Croatia) and nearby, the lateral teeth were smooth, considering that the original description of the lateral teeth was mistaken. According to this statement and based on the fact that *R. zavodniki* showed a colour pattern variation (black and red-brown to pale orange-brown), Schmekel and Cappellato (2002) considered *Runcina zavodniki* to be junior synonym of *R. ferruginea*. Our molecular and morphological analyses indicate that the Croatian specimens are distinct from *R. ferruginea* from southern England, where this species was originally described (Fig 1; Table 4). Externally, the colour pattern became now distinctive. Thus, while Croatian specimens range from black, reddish-brown to pale orange-brown (Fig. 2C, D), English specimen presents a homogeneous reddish colour with tiny dark spots all over the body usually only appreciate under the microscope (Fig. 2A, B). Internally, the radula and reproductive system are very different (Figs 4A, B and 5A, B). Concerning the radula, the rachidian tooth differs in shape and number of denticles of the pads (Fig 3A, D and Table 4). In our specimen of *R. ferruginea* from southern England, the number of denticles differs slightly from the original description where each pad possessed 10 denticles (Kress, 1977). Respect to *R. zavodniki*, although neither Thompson (1980) nor Thompson and Brodie (1988) mentioned the number of denticles of the radular teeth, our reddish Croatian specimens present denticulate lateral teeth (Fig. 3E), as mentioned in the original description of *R. zavodniki* (Thompson, 1980). However, the English *R. ferruginea* present smooth lateral teeth (Fig. 3B).

Kress (1977) presented a well description of the reproductive system of *R. ferruginea* based on histological serial sections, as well as in posterior studies where she presented detail histological descriptions of female and male organs (Kress, 1985, 1992). Neither Thompson (1980) nor Thompson and Brodie (1988) mentioned any detail about the reproductive system of *R. zavodniki*. In the present study (Figs 4A, B), we observed that the female part is divided in three lobes, but in *R. ferruginea* this part is wider than in Croatian specimens. The male part organ shows some differences. The prostate gland is broadly rounded on the posterior part and slightly narrower towards the male opening in Croatian specimens, while in *R. ferruginea* it is mostly cylindrical. Usually, the posterior end of the prostate opens into the seminal vesicle, like in *R. ferruginea*, but we observed that the opening to the seminal vesicle in *R. zavodniki* occurs at the second third of the length of the prostate (Figs 5A, B). Due to these significant differences respect to *R. ferruginea* and that our Croatian specimens match the original description of *R. zavodniki*, we concluded that these two species are not conspecific as suggested by Schmekel and Cappellato (2002), but two valid distinct species.

The other two new species within the "*Runcina ferruginea*" complex revealed by our study are, in fact, very similar externally to *Runcina ferruginea* (Fig. 3A, D, E) showing all of three a reddish to reddish-brown colour pattern. Nevertheless, *R. ferruginea* and *Pseudoruncina marinae* sp. nov. are the only reddish species possessing small dark spots distributed all over the body (Table 4). Schmekel and Cappellato (2002) also observed the small dark spots all over the body and the dark line on the notum edge in all four specimens of *R. ferruginea* from Banyuls-sur-Mer, France (Mediterranean).

While externally the two new species and *R. ferruginea* are very similar, internally they differ considerably from each other. Both *R. ferruginea* and *R. lupiaensis* sp. nov. have the lateral teeth smooth but differ in shape. The pads of the rachidian teeth also differ in shape and number of denticles (Fig. 3A, G; Table 4). *Pseudoruncina marinae* sp. nov. differs drastically from all "reddish *Runcina*" by having no radula. The specimens of *R. ferruginea* collected by Schmekel and Cappellato (2002) in Banyuls-sur-Mer (France, Mediterranean Sea) present radula (24 x 1.1.1) differing from our specimens of *Pseudoruncina marinae* sp. nov. from the same locality. Concerning to the reproductive system, the differences between the three species are mainly related with the shape and size of the organs (Figs 4 and 5). The female and male organs from our *R. ferruginea* specimen are very alike to those from the original description (Kress, 1977). Neither Cervera, García-Gómez and García (1991) nor Schmekel and Cappellato (2002) mentioned any aspect of the reproductive system of *R. ferruginea*. An interesting shape is observed in the male organ of *Pseudoruncina marinae* sp. nov., where the prostate gland is strongly curved in two points forming an "S" lying down opening into a seminal vesicle pickle-shaped (Fig. 5D).

The Canarian "reddish *Runcina*" (*R. hidalgoensis*, *R. palominoi* and *R. medanensis*) show some differences if compared with our "*R. ferruginea"* complex species (Table 4). Respect to the colour pattern and radula, the species *R. palominoi* is very distinctive by showing white spots and blotches on the notum and ventral surface of the foot; and by having the rachidian teeth unicuspid with stretch marks (Table 4). Concerning the reproductive system, the male copulatory organ of all three Canarian species differs completely to those described in this study. In *R. hidalgoensis*, for example, the seminal vesicle is separated from the prostate gland by an elongated duct and its size correspond to the prostate gland and penial papilla together (Ortea and Moro, 1999, fig. 4F) while in *R. medanensis* the seminal vesicle is very small (Ortea and Moro, 1999, fig. 2F). The only detail about the male organ of *R. palominoi* is that the seminal vesicle is folded over the prostate. Ortea and Moro (1999) do not mention any aspect of the female part of the Canarian species.

The new taxonomy of *Runcina ferruginea* presented in this study shows that the two new species described here are restricted to Mediterranean Sea, while *R. ferruginea* proper has widespread distribution from England (Atlantic) to France (western Mediterranean) (Fig. 8). So far, *R. zavonidki* and *R. lupiaensis* sp. nov. are only known from Croatia and southern coast of Italy, respectively. The species *Pseudoruncina marinae* sp. nov. is restricted to western Mediterranean (Spain and France) where it overlaps with *R. ferruginea* proper.

Due to their small size, runcinids are hard to be observed/find and, consequently, a more detailed study of internal morphology not always is possible because of the small number of specimens available. Therefore, many identifications are based on colour patterns from well-established species. However, the use of molecular markers has shown that the diversity existent among runcinids goes far beyond colour pattern and even radular morphology.

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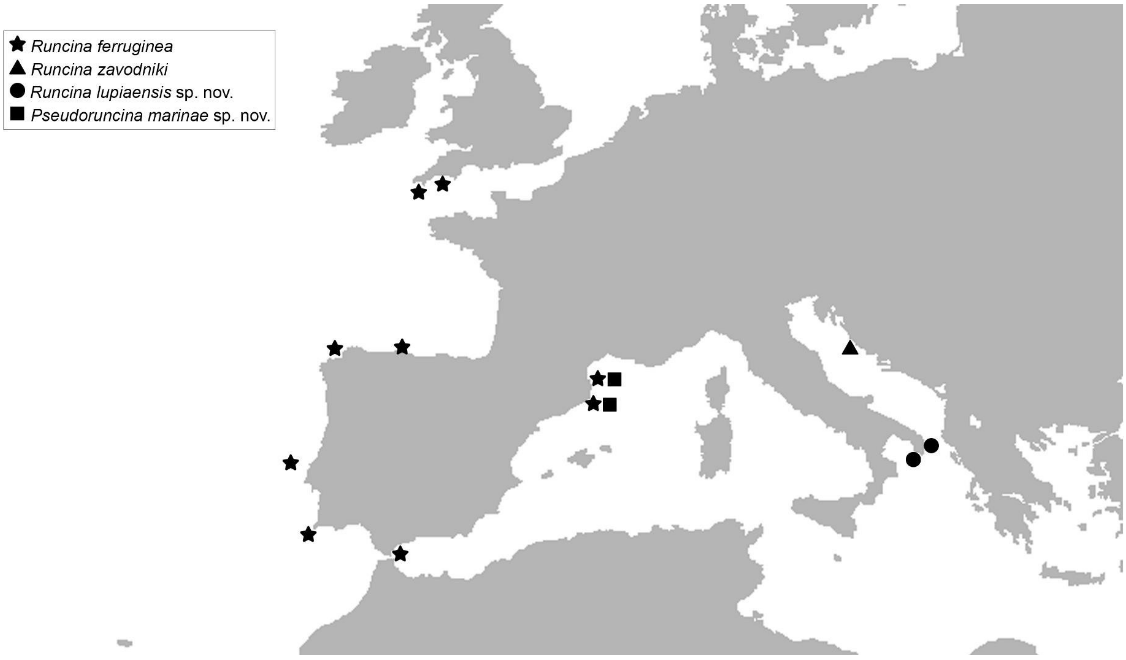

Figure 8. Distribution of the different species of *"Runcina ferruginea"* species complex.

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Table 1. List of specimens used for phylogenetic analysis. (*) Sequences from Chapter I. ([1]) Names proposed in the Chapter I. Museum abbreviations: Museum Victoria collections (NMVF), University Museum of Bergen (ZMBN), Zoologische Staatssammlung München, Germany (ZSM), Museo Nacional de Ciencias Naturales (MNCN) and The Natural History Museum, London, United Kingdom (NHMUK).

Species	New taxonomic assignment after phylogenetic study	Locality	Voucher no.	H3	COI	16S
Ilbia ilbi Burn, 1963		Hamers Haven, Australia	NMVF234189	MK322992	-	MK323019
Lapinura divae (Ev. Marcus and Er. Marcus, 1963)		Bermuda (GB)	ZMBN 82997	KJ022893	KF992195	KJ022825
Metaruncina setoensis (Baba, 1954)		Okinawa, Japan	ZMBN 88231	-	*	*
Runcina adriatica T. Thompson, 1980		Ugljan Island, Croatia	MNCN 15.05/88100	MK323022	MK322967	MK322996
Runcina adriatica T. Thompson, 1980		Zut Island, Croatia	MNCN 15.05/88101	-	MK322968	MK322997
Runcina brenkoae T. Thompson, 1980		Split, Croatia	MNCN 15.05/88086	MK322998	MK322969	MK323023
Runcina brenkoae		Nin, Croatia	MNCN 15.05/88088	MK323018	MK322971	MK323039
Runcina coronata (Quatrefages, 1844)		Swanage, England	MNCN 15.05/88105	MK323010	MK322976	MK323031
Runcina caletae[1]		Cádiz, Spain	Pending number	*	*	*
Runcina ferruginea Kress, 1977		Newlyn, Cornwall, England	MNCN 15.05/88109	MK323011	MK322977	MK323032

Table 1. Continued.

Species	New taxonomic assignment after phylogenetic study	Locality	Voucher no.	H3	COI	16S
Runcina ferruginea	***Runcina lupiaensis* sp.nov.**	Lecce, Italy	Pending number	*	*	-
Runcina ferruginea	***Runcina lupiaensis* sp.nov.**	Lecce, Italy	Pending number	-	*	-
Runcina ferruginea	***Runcina lupiaensis* sp.nov.**	Lecce, Italy	Pending number	*	*	-
Runcina ferruginea	***Runcina lupiaensis* sp.nov.**	Lecce, Italy	Pending number	-	*	-
Runcina ferruginea	***Runcina lupiaensis* sp.nov.**	Lecce, Italy	Pending number	-	*	-
Runcina ferruginea	***Runcina zavodniki* Thompson, 1980**	Iz Island, Croatia	Pending number	*	*	*
Runcina ferruginea	***Runcina zavodniki***	Ugljan Island, Croatia	Pending number	*	*	*
Runcina ferruginea	***Runcina zavodniki***	Split, Croatia	Pending number	-	*	*
Runcina ferruginea	***Pseudoruncina marinae* sp. nov.**	Roses, Spain	Pending number	-	*	*
Runcina ferruginea	***Pseudoruncina marinae* sp. nov.**	Banyuls-sur-Mer, France	Pending number	*	*	-
Runcina flavonigra[1]		Cádiz, Spain	MNCN 15.05/88107	MN057639	MN057640	MN057642
Runcina avellana Schmeckel and Cappellato, 2001		Mataró, Spain	MNCN 15.05/88108	MK32300	MK322973	MK323028
Runcina hornae Schmeckel and Cappellato, 2002		Palamós, Spain	MNCN 15.05/88110	-	MK322978	MK323033

Table 1. Continued.

Species	New taxonomic assignment after phylogenetic study	Locality	Voucher no.	H3	COI	16S
Runcina hornae		Cadaqués, Spain	MNCN 15.05/88104	MK323005	MK322988	-
Runcina lusitanica Araujo, Pola, Malaquias and Cervera, 2019		Faro, Portugal	MNCN 15.05/200065	MK323002	MK322981	-
Runcina lusitanica		Faro, Portugal	Pending number	*	*	*
Runcina marcosi Araujo, Pola, Malaquias and Cervera, 2019		Roses, Spain	MNCN 15.05/88098	MK323000	MK322982	MK323025
Runcina marcosi		Banyuls-sur-Mer, France	ZSM MOL 201442089	MK322995	MK322991	MK323021
Aplysia dactylomela Rang, 1828		Cape Verde (GB)	NHMUK 20030795/ 20030796	KJ022921	KF992168	KJ022798
Micromelo undatus (Bruguière, 1792)		Tenerife, Canary Island (GB)	NHMUK 20030800	KJ022944	DQ974653	KJ022778

Table 4. Differences among "red *Runcina*" species. Data after Kress (1977), Thompson (1980), Thompson and Brodie (1988), Gosliner (1990), Ortea and Moro (1999), Schmekel and Cappellato (2002) and present study. (?): information not available; (-): does not apply.

	Runcina ferruginea	*Runcina zavodniki*	*Runcina lupiaensis* sp. nov.	*Pseudoruncina marinae* sp. nov.
Colour pattern	Homogeneous reddish. Tiny dark spots all over the body. Fine dark line on the margin of the notum and foot.	Dark, orange or brownish. Fine dark line on the margin of the notum and foot.	Homogeneous reddish-brown. Fine dark line on the margin of the notum and foot.	Reddish colour covering the notum and anterior margins of the foot. Tiny dark spots all over the body. Fine dark line on the margin of the notum.
Gills	Three reddish pinnate.	Three pinnate. Same colour of the body.	Three reddish-brown pinnate.	Three small reddish pinnate.
Shell	Absent	Absent	Absent	Absent
Radular formulae	20 x 1.1.1	20–29 x 1.1.1	23–27 x 1.1.1	-
Rachidian tooth	Bilobed. Two pads curved on the top with 15 long denticles laminae-shaped; one inner and outer undeveloped denticles.	Bilobed. Two flat pads with 8–10 long denticles laminae-shaped disposed as an arc; same size and sharp tip. One to three very small denticles in layers between the pads.	Bilobed. Two flat and short pads with 7–10 thin, same sized denticles laminae-shaped. One small denticle between pads; in some rows two denticle in layers.	Absent
Lateral teeth	Smooth, elongate, hooked-shape. Apical cusp strongly curved. Inner part of the basal region more developed.	Triangular with curved tweezers shape bearing about 20 small, pointed denticles.	Smooth, elongate, swan neck shaped.	Absent
Gizzard plates	Four with 7–8 crests	Four with 6–8 crests	Four with 10 crests	Four with 7 crests

Table 4. (Continued).

	Runcina medanensis	*Runcina palomioi*	*Runcina hidalgoensis*
Colour pattern	Homogeneous almost dark red. Notum darker than the foot.	Reddish colour. Two white patches behind the eyes. White fragmented arc at posterior notum end. White thick line on foot edge. Bluish white patches in each lobe of the head.	Light to very dark red, rose-tile.
Gills	Not observed	Three beige large laminae.	One to three
Shell	?	?	?
Radular formulae	15–16 x 1.1.1	30 x 1.1.1	13–14 x 1.1.1
Rachidian tooth	Bilobed. Two pads with 7 thick denticles; fourth denticle more developed.	Unicuspid with stretch marks. Denticles absent.	Bilobed. Two pads with 7–9 denticles; central denticles larger, decreasing to the edges; small denticle between pads.
Lateral teeth	Elongated, hooked shape bearing more than 40 long, pointed denticles.	Smooth, elongate, hooked shape.	Denticulate, elongated, hooked shape.
Gizzard plate	Four with 8 crests	Four with 8–9 crests	Four with 8–9 crests

SUPPLEMENTARY FIGURES

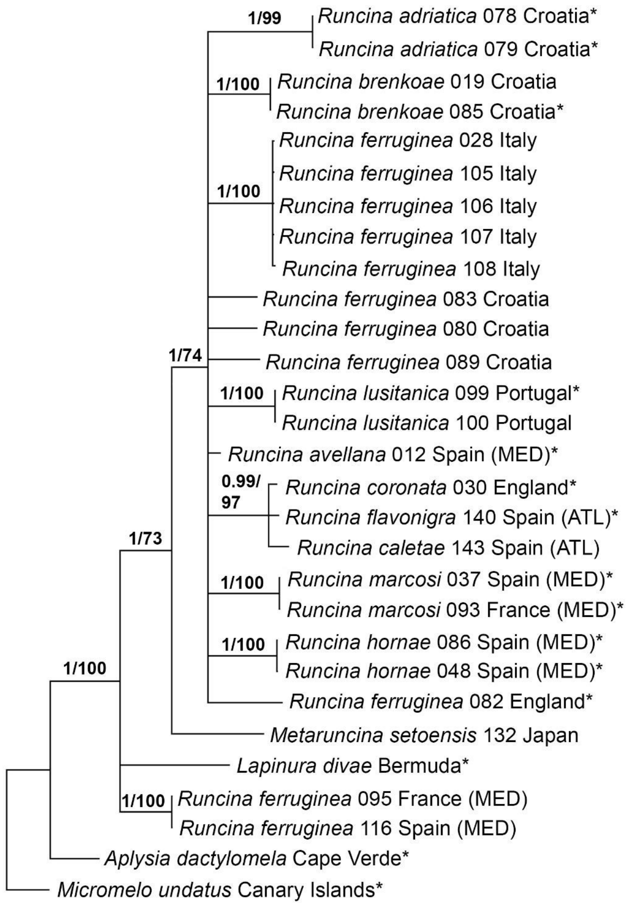

Figure S1. Phylogenetic hypothesis of "*Runcina ferruginea*" complex based on BI of the COI gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

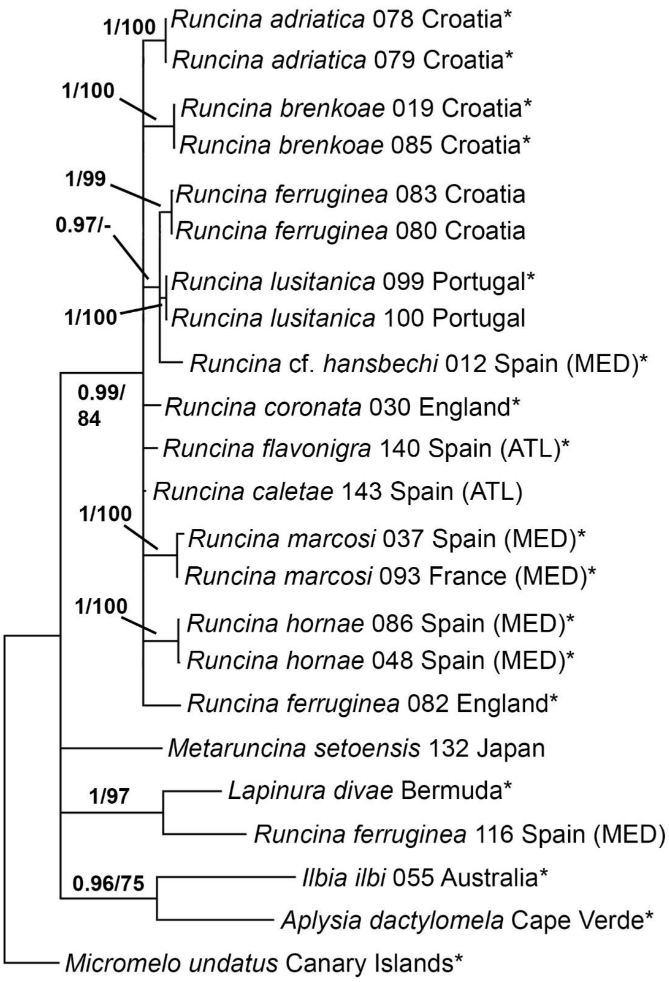

Figure S2. Phylogenetic hypothesis of "*Runcina ferruginea*" complex based on BI of the 16S gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

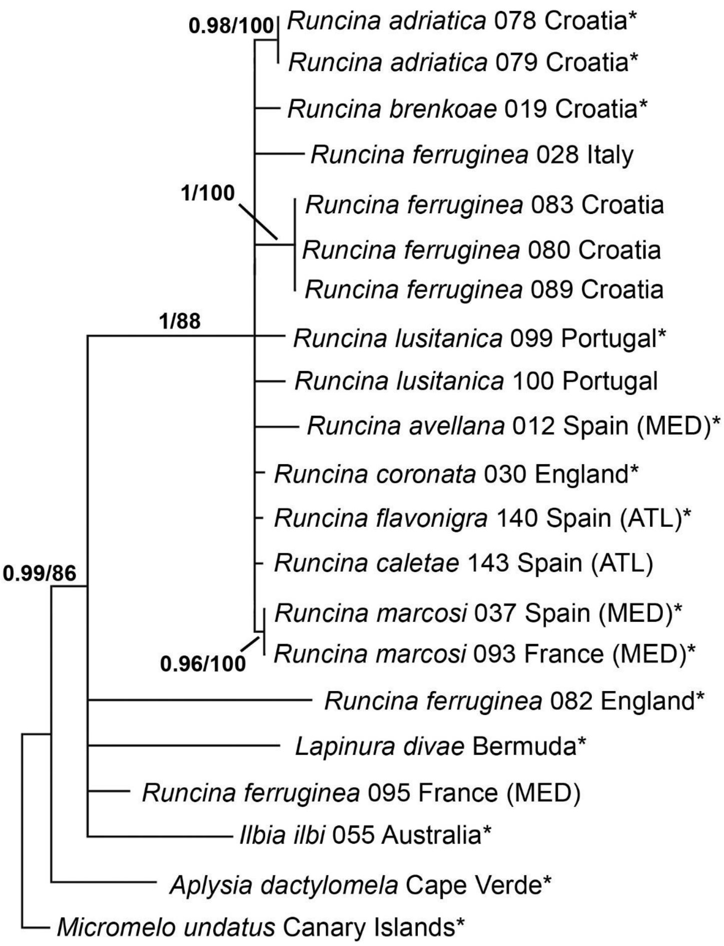

Figure S3. Phylogenetic hypothesis of "*Runcina ferruginea*" complex based on BI of the H3 gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

Capítulo 4

Does the colour pattern really matter? An integrative taxonomical approach to the case of *Runcina adriatica* Thompson, 1980

Declaración: Esta obra no se considera publicada a los efectos previstos en el Art. 8.2 del Código Internacional de Nomenclatura Zoológica. No constituye, por tanto, un registro científico público y permanente para fines de nomenclatura zoológica.

Does the colour pattern really matter? An integrative taxonomical approach to the case of *Runcina adriatica* Thompson, 1980

ABSTRACT

In runcinids, the colour pattern has been primordial for a preliminary identification, although it can sometimes lead to a misidentification. In the Chapter I, our phylogenetic analyses revealed that several specimens were misidentified as known species based on colour pattern. Among these known species, *Runcina adriatica* Thompson, 1980, described from Adriatic Sea, shows a remarkable colour pattern with an anterior and posterior horizontal white band connected by a longitudinal white line on the notum, besides numerous brown patches on it. In this chapter, we have analysed specimens from Portugal (Azores Islands, Atlantic Ocean), central and western Mediterranean Sea (Spain, Italy and Croatia) early identified as *R. adriatica*. Since the results in Chapter I revealed the species *R. adriatica* as species complex, the goal of this study is to assess the taxonomic value of the colour in *R. adriatica* using molecular phylogenetics, species delimitation analyses (ABGD, bPTP) and morpho-anatomical characters. Our results reveal that specimens early identified as *R. adriatica* and possessing the same colour pattern can belong to different species, but also specimens with different colour pattern can be conspecific. Therefore, in the present chapter, we identify and describe five distinct species belonging to "*R. adriatica"* species complex: *R. adriatica* proper, *R. lucidum* sp. nov., *R. tiramisu* sp. nov., *R. azorica* sp. nov. and *Runcina* sp.

INTRODUCTION

First described from Croatia (Adriatic Sea), the species *Runcina adriatica* has been reported in Portugal (Gosliner, 1990, Malaquias *et al*. 2009b, 2014 [Azores Islands]); Spain (Cervera *et al*., 2004 [Canary Islands], García-Gómez et al., 2011 [eastern Andalusia], Ballesteros *et al*., 2016 [Catalonia, northeastern coast]); France [Mediterranean coast] and Naples, Italy (Schmekel and Cappellato, 2002). *Runcina adriatica* is characterized by a flattened and quadrangular body, a median posterior pallial crest and a translucent white ground colour with numerous brown patches and chalk-white specks on the notum. These white specks form a median longitudinal line, which is connected with horizontal white band placed on the anterior part of the body behind the eyes and posteriorly with that on the pallial crest (Thompson, 1980, pp. 154–157, figs 1a, b). Additional material of *R. adriatica* from Croatia were collected by Thompson and Brodie (1988) and some differences were observed respect to the type material: the median crest and median white line was absent in the smallest individuals;

the larger individuals showed three distinct gills while the juveniles showed only 1 or 2 gills. Despite that, the larger specimens possessed the typical features described in the original description.

In these minuscule heterobranch sea slugs, the colour pattern is primordial for a preliminary identification, more than any other external features. The colouration in runcinids results from two main sources, the colour of the internal structures and the pigmentation of the epidermis. However, it was demonstrated that small differences in the same general colour pattern may hide the occurrence of cryptic/pseudocryptic species (see Chapter I, Araujo *et al.*, 2019 [Chapter II], Chapter III). In the present Chapter, we carry out an integrative taxonomical approach of the species *Runcina adriatica*. We assess the taxonomic value of the colour pattern in this species by testing if the same colour pattern hides different species or if the same species could show different colour pattern.

MATERIAL AND METHODS

DNA extraction, amplification and sequencing

All specimens used in this study were those obtained in Chapter I and from Genbank (Table 1). *Micromelo undatus* (Bruguière, 1792) was used to root the tree as outgroup taxon. Tissue samples were taken from the foot and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). DNA extraction, amplification and sequencing follow Chapter I of this Thesis

Specimens from the type locality, Red Island, San Andrea (Croatia) could not be obtained, but we sequenced specimens from nearby localities, Iz, Zut and Ugljan Islands mean distance 169.21 km away to south from Red Island.

Phylogenetic analyses

Sequences were edited in Geneious v10.2.3 (Drummond *et al.* 2009) and aligned using MAFFT (Katoh *et al.*, 2009) implemented in Geneious v10.2.3 (Drummond *et al.* 2009) with the default settings (Auto [FFT-NS-1, FFT-NS-2, FFT-NS-i or L-INS-i; depends on data size]). Sequences from the protein-coding genes COI and H3 were translated into amino acids to check for stop-codons. Hypervariable regions of the 16S alignment where homology could not be confidently established were removed using Gblocks under relaxed settings (Talavera and Castresana, 2007). Nevertheless, analyses including and excluding these regions provided similar results. Therefore, final analyses were performed including all bases. Sequences of the COI, 16S and H3 genes were trimmed to 658, 465 and 328 nucleotides, respectively. All three genes were concatenated using Mesquite v3.2 (Maddison and Maddison, 2018), resulting in a final dataset of 1451 base pairs. Single gene and concatenated (H3+COI+16S) analyses were performed. Saturation for the first, second and third codon positions of the COI and H3 genes were calculated in MEGA v7.0 (Kumar *et al*., 2016).

The best-fit evolutionary model for each gene was determined in jModeltest v2.1.6 (Guindon and Gascuel, 2003; Darriba *et al*., 2012), under the Akaike information criterion (AIC) (Akaike, 1974). The GTR + G + I model was selected for all three genes. Bayesian inference (BI) analyses were performed in MrBayes v. 3.2.1 (Ronquist and Huelsenbeck, 2003) with a random starting tree and two parallel runs of 10^7 generations. Convergence was checked in TRACER v1.7.1 (Rambaut *et al*., 2018) with a burn-in of 25%. Nodes with a posterior probability (PP) ≥ 0.95 (Alfaro *et al.*, 2003) were considered well supported and discussed. Maximum likelihood (ML) analysis was executed using RAxML v8 (Stamatakis, 2014) and node support was assessed with nonparametric bootstrapping (BS) with 5000 replicates. Nodes with bootstrap values (BS) ≥ 70 (Hillis and Bull, 1993) were considered significant and were discussed. Both BI and ML trees were visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Minimum and maximum pairwise uncorrected *p*-distances of COI within and between species were calculated in MEGA v7.0 using all sequences available (Kumar *et al*., 2016).

Species delimitation analyses

The Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al*., 2012) and the Bayesian Poisson tree processes (bPTP) (Zhang *et al*., 2013) analyses were used to aid delimiting species. For the ABGD we used the alignment from the fast-evolving COI gene with default settings ($P_{\min} = 0.001$, $P_{\max} = 0.1$, Steps = 10, $X = 1.2$, Nb bins = 20), under the three models of evolution available, namely Jukes-Cantor (JC69), Kimura (K80) and Simple Distance. The bPTP analysis is an updated version of the original maximum likelihood PTP (modelling speciation in terms of the number of substitutions), which adds Bayesian support values to delimit species. The bPTP analyses were run with the COI and 16S trees using the webserver (https://species.h-its.org/ptp/) (Zhang *et al*., 2013).

Morphology

Specimens early identified as *Runcina adriatica/R.* cf. *adriatica* from Azores Islands (Portugal) (2 specimens), Catalonia (Mediterranean, northeastern Spain) (5 specimens), Otranto, (southeastern Italy) (3 specimens) and Croatia (6 specimens) were studied anatomically to complete and compare the results obtained by molecular phylogenetics and species delimitation analyses. We also included one specimen previously identified as *Runcina* *hornae* Schmekel and Cappellato (2002) and *Runcina* sp., because in the phylogenetic analysis from Chapter I these specimens clustered together with specimens identified as *R.* cf. *adriatica*.

Animals were dorsally/ventrally dissected and the buccal bulb was extracted and dissolved in a solution of 10% sodium hydroxide to expose the radula. The radula and gizzard plates were then immersed in water, dried and mounted for scanning electron microscopy (SEM) with a Nova NanoSEM 450 available at the University of Cadiz (Cadiz, Spain). The reproductive systems were examined and drawn using a dissecting microscope with the aid of a *camera lucida.*

RESULTS

Phylogenetic and species delimitation analyses

The combined tree (H3+COI+16S) provided better resolution than H3, COI or 16S separately (Fig. 1, and Supplementary Material Figs S1, S2 and S3). The topologies of the ML trees were congruent with those obtained from Bayesian analyses, although bootstrap values were lower than the posterior probabilities in same species clades (ML trees not shown). No saturation was observed.

Figure 1 shows that *Ilbia ilbi* Burn, 1963 was rendered sister to clade I (red bar) (PP = 1; BS = 98). Within clade I, *Lapinura divae* (Er. Marcus and Ev. Marcus, 1970) was sister to clade II (yellow bar) (PP = 0.97; BS = 76). *Metaruncina setoensis* resulted to be sister lineage of the genus *Runcina* (grey square), however, this relationship was recovered only by BI analysis (clade II; PP = 0.99; not recovered in ML).

In this study, *Runcina* specimens clustered together in a monophyletic and well-supported clade (PP = 1; BS = 85). The specimens previously identified as *R. adriatica* or *R.* cf. *adriatica* split into five well-supported subclades, which we called groups A, B, C, D and E. Group A included specimens from Azores (Portugal) and Canary Islands (Spain); group B was composed of specimens from Croatia; group C contained specimens from southeastern Italy; group D comprised specimens from Catalonia (northeastern Spain), Croatia and SE Italy; and group E included only one specimen from southeastern Italy (Fig. 1).

The ABGD analysis with all three models of evolution resulted in 18 groups, five of them corresponding to the same *R. adriatica* or *R.* cf. *adriatica* clades recovered in the BI and ML analyses (Fig. 1A). The results obtained with the bPTP analysis showed evidence of over-splitting, dividing the *R. adriatica* (group D) into three different groups (Fig. 1B). The minimum uncorrected *p*-distance (Table 2) for COI among all five groups ranged from 9.1% to14.4%. The maximum distance within the group A was 0.8%, in the group B was 1.2%, in the group C was 0.9% and within group D was 4.2%. The group E had one single specimen.

Our molecular results, backed by morphological differences, identified the existence of five species under the name "*Runcina adriatica*" or "*R.* cf. *adriatica*". These species are formally redescribed or described in the Systematic section below.

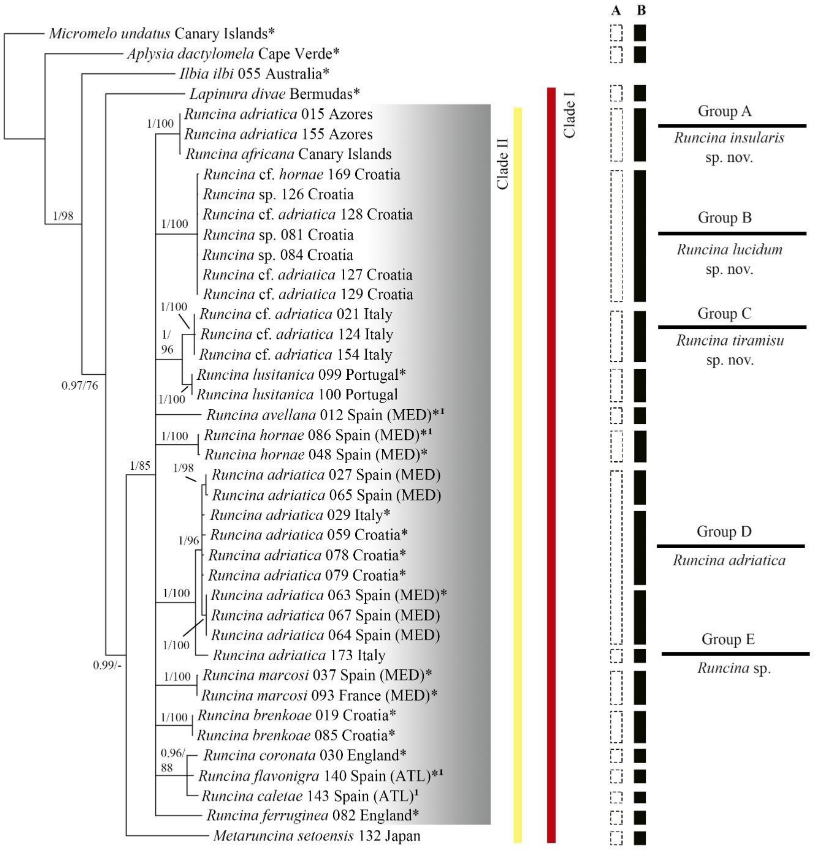

Figure 1. Phylogenetic hypothesis for the "*Runcina adriatica*" complex based on the combined dataset (H3+COI+16S) inferred by Bayesian analysis. Numbers on the left of the slashes are posterior probabilities. Numbers on the right are bootstrap values from Maximum Likelihood. Abbreviations: ATL, Atlantic Ocean; MED, Mediterranean Sea. (**A**) ABGD results based on the COI data set. (**B**) bPTP results based on the COI data set. (*) Refers to sequences from Genbank. (1) Refers to names proposed in Chapter I.

Table 2. Uncorrected *p*-distances based on COI sequences between "*Runcina adriatica*" complex groups.

	Distance between groups (%)				Distance within groups (%)
	Group A	Group B	Group C	Group D	
Group A					0.7–0.8
Group B	10.6–11.4				0.2–1.2
Group C	9.8–10.9	11.2–12.5			0.3–0.9
Group D	11.5–12.8	11.6–13.7	13.4–14.6		0.0–4.2
Group E	12.8–13.6	12.9–13.3	14.4–14.8	9.1–9.8	-

Systematic description

Family RUNCINIDAE H. Adams and A. Adams, 1854
Runcina Forbes in Forbes and Hanley, 1851

Runcina adriatica Thompson, 1980
(Figs 2; 3A–C; 4A; 5A)

Thompson (1980): 154, fig. 1A, B; Thompson. and Brodie (1988): 340, fig. 1A, B; Gosliner (1990): 141, figs 1B, 5B, 6, 7; Schmekel and Capellato (2001): 144, Pl. I d; 145, Pl. II j; and 148, Pl. III e, f; Schmekel and Capellato (2002): 94, 95, Pl. VI a – d, Table I; Cervera *et al.* (2004): 18; Malaquias *et al.* (2009b): 4; Malaquias *et al.* (2014): 140; García-Gómez *et al.* (2011): 423; Ballesteros *et al.* (2016): 4, fig. 7A; Prkić *et al.* (2018): 121, figs 1–5, 122, figs 1, 2.

Type material: Holotype (NHMUK 197912W) Natural History Museum, London, UK (not studied because the material is only available as micro-slide preparations).

Type locality: St. Andrew Island, Croatia.

Distribution: Catalonia, northeastern coast Spain (Ballesteros *et al.*, 2016, present study); Banyuls-sur-Mer, Mediterranean coast France and Naples, Italy (Schmekel and Cappellato, 2002); Otranto, Italy (present study) and Croatia (Thompson, 1980; Thompson and Brodie, 1988; Prkić *et al.*, 2018, present study).

Examined material: Croatia: MNCN 15.05/88099, Iž Island, Croatia, Alen Petani coll., 23 March 2017, 1.5 mm in length, depth 4–8m (dissected and sequenced). MNCN 15.05/88101, Zut Island, Croatia, Alen Petani coll., 26 Aug 2017, 2.1 mm in length, depth 4–6m (dissected and sequenced). MNCN 15.05/88100, Ugljan Island, Croatia, Alen Petani coll., 30 Sep 2017, 1,5 mm in length preserved, depth 4–6m (dissected and sequenced); **Italy**: MNCN 15.05/88102, Otranto, Italy, Fabio Vitale coll., 24 May 2015, 1 mm in length preserved, depth 8 m (sequenced); **Spain**: MNCN 15.05/90416, Palamós, Catalonia, Spain, Enric Madrenas coll., 18 Sep 2015, 1 mm in length preserved, depth 5 m (sequenced). MNCN 15.05/88103, Cadaqués Catalonia, Spain, Carles Galià coll., 30 May 2017, 3 mm in length, depth 6m (sequenced). MNCN 15.05/90417, Cadaqués Catalonia, Spain, Carles Galià coll., 16 May 2017, 3 mm in length, depth 5m (sequenced). MNCN 15.05/90418, Cadaqués Catalonia, Spain, Carles Galià coll., 16 May 2017, 2.5 mm in length, depth 2m (sequenced). MNCN 15.05/90419, Cadaqués Catalonia, Spain, Carles Galià coll., 16 May 2017, 4 mm in length, depth 4m (sequenced).

External morphology (Fig. 2): Body flattened and quadrangular. Notum smooth. Foot as wide as notum. Most specimens show a developed median pallial crest at the posterior end of the notum. The head zone isosceles trapeze-shaped, slightly bilobed and narrower than the foot. Anterior part of the foot rounded and wider than the head. Foot end rounded extending posteriorly beyond the notum. Ground colour yellowish translucent. Digestive gland visible through the skin (reddish colour). Notum, mantle groove and foot covered with black or dark brown dots and white specks. On the notum, the dark dots are dispersed while the white specks are concentrated on the head anteriorly to eyes forming two oval blotches. Behind the eyes and posteriorly, on the notum edge, the white specks form horizontal bands, which are connected by a longitudinal white line. Yellowish longitudinal band with dark oval spots on the median dorsal surface of the tail. White speckles on each side of the longitudinal band. Three small plate-like gills to the right of the anus. Gills yellowish translucent with small dark and white dots. Anus located in median line of body, beneath the end of notum. Shell absent.

Internal anatomy (Figs 3A–C; 4A; 5A): Radular formulae 23 x 1.1.1 (MNCN 15.05/88100) and 33 x 1.1.1 (MNCN 15.05/90419). Rachidian tooth boomerang shaped with smooth lateral triangular wings on each side. Central part bilobed; masticatory edge possesses two pads with upper part slightly curved or flattened; each pad possesses 7–8 or even 13 long denticles laminae-shaped with same size and disposed as an arc. Central deep and broad depression is present between the pads; with a very tiny denticle in some teeth (Fig. 3A). Lateral teeth smooth, elongated and curved like a swan neck. Long basal region. Apical cusp shorter than the basal region. (Fig. 3B). Triangular jaws present. Four gizzard plates with 8–11 lamellae (Fig. 3C). Reproductive system monoaulic. Female gland mass elliptical-shaped divided into two lobes.

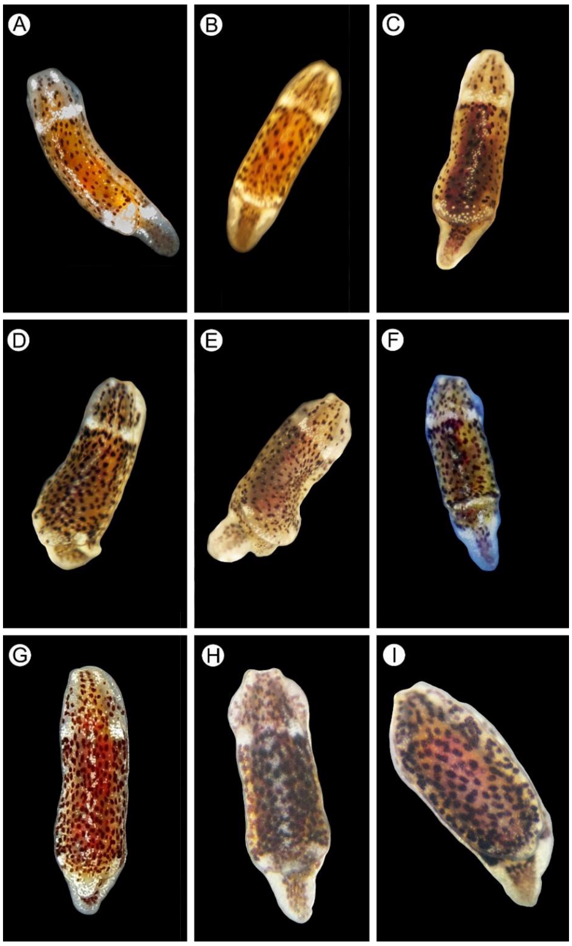

Figure 2. Living animals of *Runcina adriatica*. **A**-**E.** Specimens from Catalonia, Spain (Mediterranean) (photos: Enric Madrenas, Carlès Galiá). **A.** MNCN 15.05/90416, **B.** MNCN 15.05/88103, **C.** MNCN 15.05/90418, **D.** MNCN 15.05/90417 and **E.** MNCN 15.05/90419. **F.** Specimen from Lecce, Italy (photo: Fabio Vitale), MNCN 15.05/88102. **G**-**I.** Specimens from Croatia (Adriatic Sea) (photos: Alen Petani). **G.** MNCN 15.05/88100, **H.** MNCN 15.05/88101 and **I.** MNCN 15.05/88099.

Bursa copulatrix absent. Cylindrical common genital duct subtly curved next to female mass, connecting the female gland mass to the exterior on the posterior right side of the body (Fig. 4A). Female gland mass placed beneath the posterior right side of digestive gland. Male copulatory organ opens to the right of the mouth. Short rounded penial papilla projects into the atrium. Penial papilla unarmed. Prostate gland long and cylindrical. Long and curved seminal vesicle with pointed ending. Seminal vesicle one-third size of the prostate. (Fig. 5A).

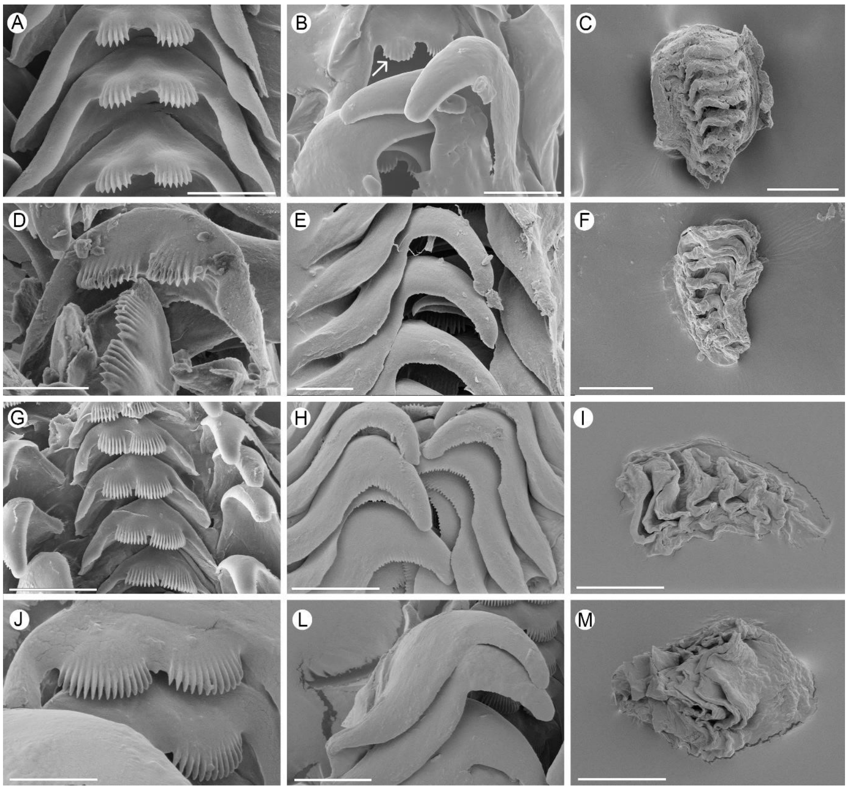

Figure 3. Scanning electron micrographs of radula and gizzard plates of *Runcina* species. **A, B, C**. *R. adriatica* (MNCN 15.05/90418, MNCN 15.05/90419, MNCN 15.05/88100). **A**. Rachidian teeth (MNCN 15.05/88100). **B**. Lateral teeth (MNCN 15.05/90419); white arrow: pad of the rachidian tooth from a Spanish specimen of *R. adriatica*. **C**. Gizzard plates (MNCN 15.05/90418). **D, E, F**. *R. lucidum* sp. nov. (MNCN pending number of voucher). **J.** Rachidian teeth (MNCN pending number of voucher). **L**. Lateral teeth (MNCN pending number of voucher). **G, H, I**. *R. tiramisu* sp. nov. (MNCN pending number of voucher). **G**. Rachidian teeth. **H**. Lateral teeth. **I**. Gizzard plates. **J, L, M**. *R. insularis* sp. nov. (MNCN pending number of voucher) **D**. Rachidian tooth. **E.** Lateral teeth. **F**. Gizzard plates. **M**. Gizzard plates (MNCN pending number of voucher). Scale bars: **A, D, L** = 5 µm; **B, E, G, H** = 10 µm; **C, F, I, M** = 50 µm; **J** = 4 µm.

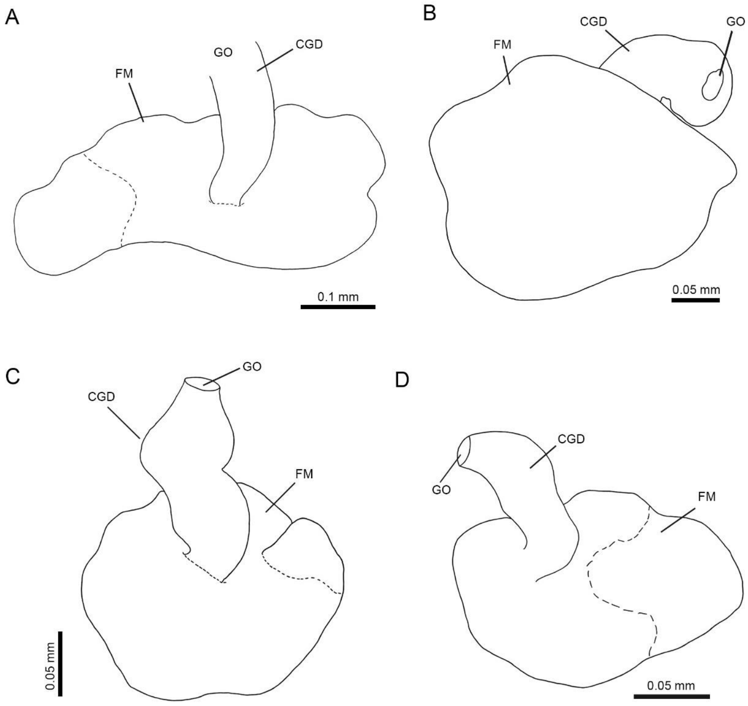

Figure 4. Female reproductive system of *Runcina* species. **A**. *R. adriatica* Croatia (Adriatic Sea) (MNCN 15.05/88099). **B**. *R. lucidum* sp. nov., Croatia (Adriatic Sea) (MNCN pending number of voucher). **C**. *R. tiramisu* sp. nov., Lecce, Italy (MNCN pending number of voucher). **D**. *R. insularis* sp. nov., Azores Islands, Portugal (MNCN pending number of voucher). Abbreviations: FM, female mass; CGD, common genital duct; GO, gonopore.

Runcina lucidum **sp. nov.**

(Figs 3D–F; 4B; 5B; 6)

Runcina sp. 1, Prkić *et al*. (2018): 124, figs 1, 2.

Runcina sp. 2, Prkić *et al*. (2018): 124, figs 3, 4.

Runcina sp.3, Prkić *et al*. (2018): 125, figs 1, 2.

Examined material: Holotype: MNCN (pending number of voucher), Zut Island, Croatia, Alen Petani coll., 06 sep 2017, 5 mm in length, depth 1–3m (sequenced and dissected). Paratype: MNCN (pending number of voucher), Zut Island, Croatia, Alen Petani coll., 26 Aug 2017, 1 mm in length preserved, depth 4–6m (sequenced and dissected). Additional materials: MNCN (pending number of voucher), Iz Island, Croatia, Alen Petani coll., 13 Aug 2017, 2 mm in length, depth 7–16m (sequenced). MNCN (pending number of voucher), Ugljan Island, Croatia, Alen Petani coll., 06 sep 2017, 6 mm in length, depth 1–3m (sequenced). MNCN (pending number of voucher), Zut Island, Croatia, Alen Petani coll., 26 Aug 2017, 4 mm in length, depth 4–6m (sequenced). MNCN (pending number of voucher), Zut Island, Croatia, Alen Petani coll., 26 Aug 2017, 1.2 mm in length preserved, depth 4–6m (sequenced). MNCN (pending number of voucher), Zut Island, Croatia, Alen Petani coll., 06 sep 2017, 4 mm in length, depth 1–3m (sequenced and dissected).

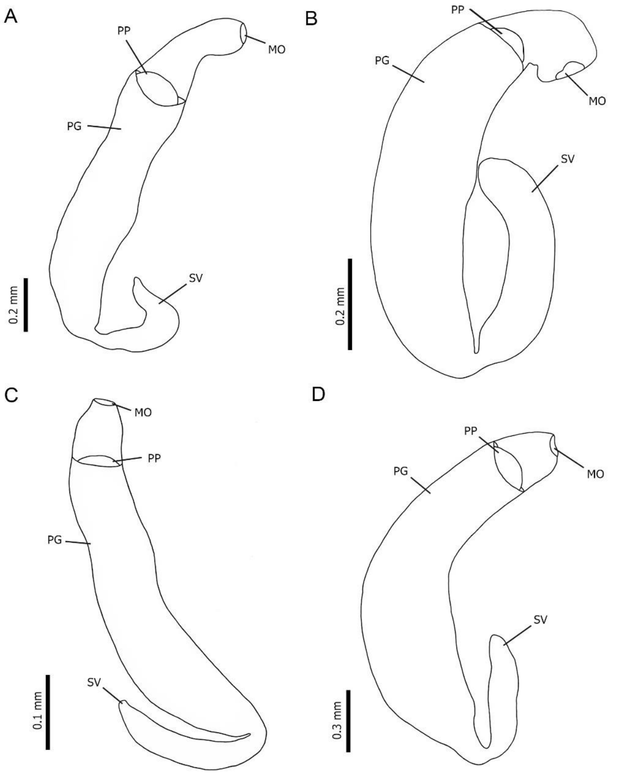

Figure 5. Male reproductive system of Runcina species. **A**. *R. adriatica* Croatia (Adriatic Sea) (MNCN 15.05/88100). **B**. *R. lucidum* sp. nov., Croatia (Adriatic Sea) (MNCN pending number of voucher). **C**. *R. tiramisu* sp. nov., Lecce, Italy (MNCN pending number of voucher). **D**. *R. azorica* sp. nov., Azores Islands, Portugal (MNCN pending number of voucher). Abbreviations: AT, atrium; MO, male opening; PP, penial papilla; PG, prostate gland; SV, seminal vesicle.

Etymology: The specific name *lucidum* refers to the shiny effect of the chalk white spots dispersed all over the body.

Distribution: Midwest Croatia (present study).

External morphology (Fig. 6): Elongated and flattened body, almost rectangular. Notum smooth. Head with small lobes in the front. Notum sometimes pointed on the posterior end and slightly narrow towards the head. Notum as wide as foot. Anterior part of the foot rounded and wider than the notum in some specimens (Fig 6B, D). Foot extends far beyond the notum, sometimes rounded or pointed. Ground colour grey, light brown or orange-honey. Specimens with light brown colour bear a pattern of anastomosing brown blotches on notum and lateral margins of the foot. In specimens with grey colour the brown blotches are dispersed. Brown blotches absent in orange-honey specimens. Chalk white spots all over the body, more concentrated on posterior margin of the foot and posterior end of the notum. Two oval white blotches on each side behind eyes. Horizontal band of white spots on the surface of the notum, behind eyes. Orange-honey specimens bear a discontinuous white line and continuous dark brown line on lateral margins of the notum. Eyes visible. Ventral surface of foot translucent with white spots. Longitudinal band of the same colour of the body on the dorsal surface of foot. Three small translucent plate-like gills to the right of anus. Anal pore located in median line of body, beneath the end of notum. Shell absent.

Internal anatomy (Figs 3D–F; 4B; 5B): Radular formulae 22 x 1.1.1 (MNCN pending number of voucher) and 25 x 1.1.1 (MNCN pending number of voucher). Rachidian tooth boomerang shaped with smooth lateral wings on each side. Central part bilobed; masticatory edge contains a pair of flattened pads; each pad possesses around 7–10 relatively long, sharped denticles laminae-shaped with the same size and disposal straight. Central small depression is present between the pads; small denticle absent (Fig. 3D). Lateral teeth smooth, elongated, hooked-shape. Long basal region. Apical cusp almost the same size of basal region (Fig. 3E). Triangular jaws present. Four gizzard plates with 7–8 lamellae (Fig. 3F). Reproductive system monoaulic. Female gland mass with amoeboid shape, not divided into lobes. Common genital duct short, wide and curved, connecting the female gland mass to the exterior on the posterior right side of the body (Fig. 4B). Female gland mass placed beneath the posterior right side of digestive gland. Male copulatory organ opens to the right of the mouth. Penial papilla short, unarmed and rounded projects into the atrium. Atrium wide slightly curved. Prostate gland wide, long and cylindrical. Seminal vesicle long, thick, cylindrical and curved towards the prostate. The seminal vesicle size is two thirds of the prostate (Fig. 5B).

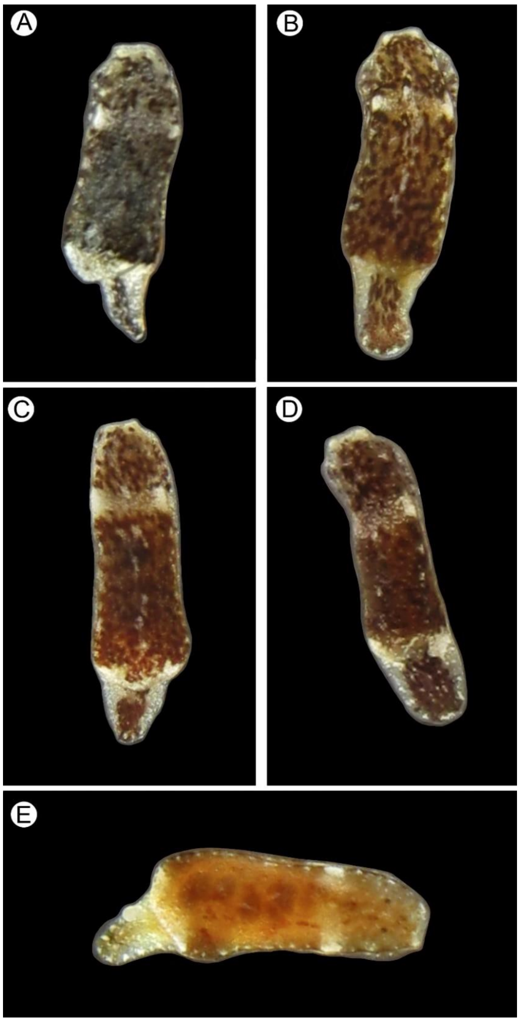

Figure 6. Living animals of *Runcina lucidum* sp. nov. **A.** MNCN (pending number of voucher), Specimens from Zut Island, Croatia (Adriatic Sea) (photo Alen Petani). **B**-**D.** Specimens from Ugljan, Croatia (Adriatic Sea) (photos Alen Petani). **B.** MNCN (pending number of voucher), **C.** MNCN (pending number of voucher) and **D.** MNCN (pending number of voucher). **E.** Specimen from Iz Island, Croatia (Adriatic Sea) (photo Alen Petani) MNCN (pending number of voucher).

Runcina tiramisu sp. nov.

(Figs 3G–I; 4C; 5C; 7A–B)

Examined material: Holotype: MNCN (pending number of voucher), La Strea, Lecce, Italy, Fabio Vitale coll., 19 March 2015, 1 mm in length preserved (sequenced and dissected). Paratype: MNCN (pending number of voucher), La Strea, Lecce, Italy, Fabio Vitale coll., 20 Feb 2015, 1 mm in length preserved (sequenced). Additional material: MNCN (pending number of voucher), Santa Maria al Bagno, Lecce, Italy, Fabio Vitale coll., 22 Nov 2014, 1 mm in length preserved, depth 1m (sequenced).

Etymology: The tiramisu is a dessert from Italy that has a chocolate brown and white/beige colours, like our new species.

Distribution: Lecce, southeastern Italy.

External morphology (Fig. 7A–B): Body oval. Notum smooth. Head straight in front. Posterior part of the notum rounded. Foot as wider as notum. Anterior and posterior part of the foot rounded. Foot extends posteriorly beyond the notum. Ground colour brown chocolate. Notum almost uniformly chocolate brown with some lighter gaps on the lateral edges and both sides of the head. Two oval white patches behind the eyes and at the posterior end of the notum. Dorsal surface of the foot chocolate brown with translucent lateral edges. Eyes difficult to discern. Three small plate-like gills to the right of the anus. Gills translucent with brown margins. Anus situated in median line of body, beneath the notum. Shell absent.

Internal anatomy (Figs 3G–I; 4C; 5C): Radular formula 26 x 1.1.1 (MNCN pending number of voucher). Rachidian tooth boomerang shaped with short smooth lateral wings on each side. Central part bilobed; masticatory edge contains a pair of pads with upper part slightly curved; each pad possesses around 8–13 long and thick denticles with the same size. Central small depression is present between the pads (Fig. 3G). Lateral teeth elongate, hooked shape with 20–25 small denticles. Basal region relatively short. Apical cusp almost same size of the basal region. (Fig. 3H). Triangular jaws present. Four gizzard plates with 7 lamellae (Fig. 3I). Reproductive system monoaulic. Rounded female gland mass slightly divided into two lobes. Median size and broad common genital duct connecting the female gland mass to the exterior at the posterior right side of the body (Fig. 4C). Female gland mass placed beneath the posterior right side of digestive gland. Male copulatory organ opens to the right of the mouth. Unarmed penial papilla projects into the atrium. Prostate gland long and cylindrical. Long and slender seminal vesicle with pointed ending, placed on the opposite side of the curved region of prostate (Fig. 5C).

Runcina azorica **sp. nov.**

(Figs 3J–M; 4D; 5D; 7C)

Runcina africana Pruvot-Fol: Malaquias *et al*. (2009a), 4
Runcina adriatica Pruvot-Fol: Malaquias *et al*. (2009b), 27

Examined material: Holotype: ZMBN81756, Faial Is., Azores Island, Portugal, Juan Lucas Cervera coll., 7 Aug 2007, 1.2 mm in length preserved, 1 m on algae wash (sequenced and dissected). Paratype: MNCN (pending number of voucher), Faial Is., Azores Island, Portugal, Juan Lucas Cervera coll., 24 Aug 2007, 1 mm in length preserved (sequenced).

Etymology: The specific name *azorica* refers to the Azores Islands, the type locality is located.

Distribution: Azores Islands, Portugal (Gosliner, 1990; Malaquias *et al*., 2009b, 2014) and Canary Islands (Malaquias *et al*., 2009a).

External morphology (Fig. 7C): Body short and oval. Notum smooth with head lobes. Rounded on the posterior end and slightly narrower towards the head. Notum wider than the foot. Anterior and posterior part of the foot rounded. Foot extends posteriorly beyond the notum. Ground colour yellowish translucent. Notum, foot and mantle groove covered with dark brown and white speckles. On the notum, dark brown speckles are highly concentrated on the median zone forming one large dark patch. Isolated dark brown spots on the lateral notum edges. Small white spots concentrated on the median zone. One horizontal white band behind the eyes and another anteriorly to the posterior notum end. Fine dark orange line covers the margin of the notum. Dark brown oval spots on the median dorsal surface of the tail. Eyes difficult to discern. Two small plate-like gills, both divided into two horizontal arcs. Gills yellowish translucent with small dark dots. Anus situated in median line of body, beneath the notum. Shell absent.

Figure 7. Living animals of *Runcina tiramisu* sp. nov. **(A–B)** and *Runcina azorica* sp. nov. **(C). A.** MNCN (pending number of voucher) and **B.** MNCN (pending number of voucher), Specimens from La Strea, Lecce, Italy (photos Fabio Vitale). **C.** MNCN (pending number of voucher), Specimen from Azores Islands, Portugal (photo Manuel Malaquias).

Internal anatomy (3J–M; 4D; 5D): Radular formulae 25 x 1.1.1 (MNCN pending number of voucher) and 24 x 1.1.1 (ZMBN81756). Rachidian tooth boomerang shaped with smooth lateral wings on each side. Central part bilobed; masticatory edge contains a pair of broad and a little curved pads; each pad possesses 13–15 long denticles laminae-shaped with same size and disposed as an arc. Central deep and broad depression is present between the pads; a small denticle present (Fig. 3J). Lateral teeth smooth, elongated and triangular with curved tweezers shaped (Fig. 3L). Triangular jaws present. Four gizzard plates with 9 lamellae (Fig. 3M). Reproductive system monoaulic. Female gland mass oval-shaped divided into two lobes. Cylindrical common genital duct curved at both ends connecting the female gland mass to the exterior on the posterior right side of the body (Fig. 4D). Female gland mass placed beneath the posterior right side of digestive gland. Male copulatory organ opens to the right of the mouth. Short rounded and unarmed penial papilla projects into the atrium. Prostate gland long, wide, and cylindrical. Long and cylindrical seminal vesicle with rounded end, shorter than half size of prostate gland (Fig. 5D).

Runcina sp.
(Fig. 8)

Distribution: Otranto, southeastern Italy.
Examined material: MNCN 15.05/90666, Otranto, Italy, 30 June 2019, coll. Fabio Vitale, 2 mm in preserved length (dissected and sequenced).

External morphology (Fig. 8A): Body flattened and rectangular. Notum smooth. Posterior end part rounded, no median pallial crest. The head zone isosceles trapeze-shaped, slightly bilobed and narrower than the foot. Foot as wide as notum. Anterior part of the foot rounded. Posteriorly pointed the tail extends beyond the notum. Ground colour white and yellowish translucent. Digestive gland visible through the skin (brown colour). Notum, mantle groove and foot covered with black dots. On the notum, the small black dots are concentrated at the head and posterior end. Large black spots occupy the central zone of the notum. Small white specks distributed longitudinally on the central part and posterior edge of the notum. Two white oval blotches behind the eyes. White bands on the lateral margins of the tail. Several black spots on the central zone of the dorsal surface of the posterior end of the foot. Two or three small plate-like gills to the right of the anus. Gills yellowish translucent with small black spots. Anus located in median line of body, beneath the end of notum. Shell absent.

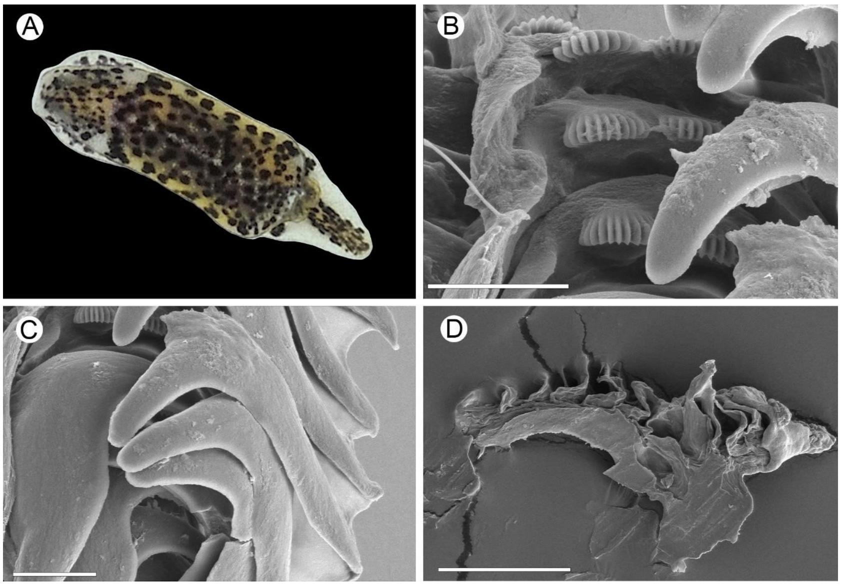

Figure 8. A. Living animal of *Runcina* sp. Specimen from Otranto, Italy (photo: Fabio Vitale), MNCN 15.05/90666; **B, C, D** Scanning electron micrographs of radula and gizzard plates. **B**. Rachidean teeth; **C**. Lateral teeth; **D**. Gizzard plates. Scale bar: **A, B** = 5 µm; **D** = 50 µm.

Internal anatomy (Fig. 8B–D): Radular formula 13 x 1.1.1 (MNCN 15.05/90666). Rachidian tooth boomerang shaped with smooth, relatively short lateral wings on each side. Central part bilobed; masticatory edge contains a pair of pads slightly curved on the upper part; each pad possesses around 10–11 relatively long denticles laminae-shaped with the same size disposed in arc. Central small depression is present between the pads; small denticle absent (Fig. 8B). Lateral teeth smooth, elongated, hooked shape with a long basal region (Fig. 8C). Triangular jaws present. Four gizzard plates with 7–8 lamellae (Fig. 8D). Reproductive system not observed, probably not developed.

DISCUSSION

For most runcinids, the colour pattern is essential for a preliminary identification. It has been considered that *Runcina adriatica* has a very characteristic colour pattern and, according to this assumption, has been recorded in the Mediterranean Sea from Croatia to southeastern coast of Spain (Thompson, 1980, Thompson and Brodie, 1988, Schmekel and Cappellato, 2002, García-Gómez *et al*, 2011, Ballesteros *et al*., 2016, Prkić *et al*. 2018) and also in Azores

(Portugal) and Canary Islands (Spain, Atlantic Ocean) (Gosliner, 1990, Cervera *et al*., 2004, Malaquias *et al*., 2009b, 2014).

Our phylogenetic analyses, with representatives from almost all areas mentioned above, except Canary Islands and France, indicate the presence of at least five species with the same or similar colour pattern to *Runcina adriatica* (Fig. 1). The ABGD analyses (Fig. 1A) also delimit the same five species while the bPTP analysis show evidence of over-splitting, with the true *R. adriatica* split into three low supported partitions (Fig. 1B, group D). Despite this over-split, the intraspecific distances for COI for these taxa range from 0.0% to 4.2%, which agree with studies that suggest that intraspecific divergences reported for molluscs are often less than 5% (Carmona *et al*., 2011, Krug *et al*., 2018, Layton *et al*., 2018). Our molecular results also show that our sequences of *R. azorica* sp. nov. and one sequence of *Runcina africana* Pruvot-Fol, 1953 from Canary Islands cluster together in a high supported subclade (group A; PP = 1; BS = 100). The ABGD and bPTP analyses showed that they are the same species with a COI uncorrected *p*-distance of 0.8%. Unfortunately, photograph of this "*R. africana*" is not available, but clearly was a misidentification. Therefore, we identify *Runcina adriatica* as a species complex, recognizing five distinct species: *R. adriatica* Thompson, 1980, *R. lucidum* sp. nov., *R. tiramisu* sp. nov., *R. azorica* sp. nov. and *Runcina* sp. Our results also support the hypotheses that specimens previously identified as *R. adriatica* and having the same colour pattern can belong to different species but also that those with different colour pattern respect to the original description can be conspecific (see Fig. 6, *R. lucidum* sp. nov.). Figure 9 shows the current distribution of *Runcina adriatica* taken from the literature (A) and the herein revised distribution of all the species within this complex (B).

Within "*Runcina adriatica*" complex, specimens of *R. lucidum* sp. nov. (Figs 1[group B] and 6), show great colour pattern variability. Despite this, the presence of oval white patches behind the eyes and white band on the posterior notum margin are consistent in all specimens examined (Table 3). Most specimens resemble the Croatian specimens of true *R adriatica* differing by the lack of the pallial crest and by the disposition and shape of the brown spots (Figs 2G, H; 6B, C, D). One specimen of *R. lucidum* sp. nov. (Fig. 6E) was previously identified as *R.* cf. *hornae* due to its extremely similarity with this French species, but our Croatian specimen differs from *R. hornae* by bearing a continuous dark brown line on lateral margins of the notum and showing brown blotches only at the notum rear. The species *R. tiramisu* sp. nov. differs from *R. adriatica* and *R. lucidum* sp. nov. by its uniformly brown colour and the existence of lighter gaps on the lateral edges and head (Fig. 7A, B; Table 3). *R. azorica* sp. nov.

differs from the Italian and Croatian species by the yellowish ground colour and from all three species by the disposition and shape of the darks spots and position of the posterior horizontal band (Fig. 7C; Table 3).

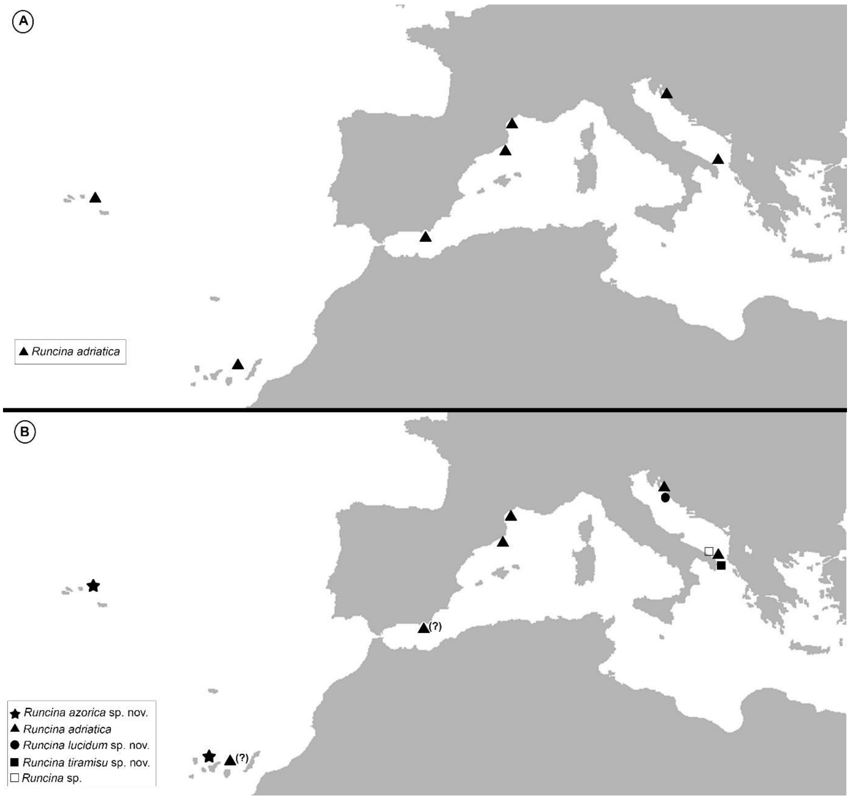

Figure 8. A. Distribution of *Runcina adriatica* according to the literature **B.** Distribution of the different species of "*R. adriatica*" species complex, including the new distribution for *R. adriatica*. The (?) indicates doubtful records.

In the original description, Thompson (1980) provided information about the radula, but no photographs or drawing were shown. Schmekel and Cappellato (2002) described several specimens identified as *R. adriatica* from Banyuls-sur-Mer (France, Mediterranean) and Naples (Italy), including SEM micrographs of the radulae, jaws and gizzard plates. The radulae of their specimens resemble those of our specimens of *R. adriatica* from northeastern Spain, which differs a little from the Croatian specimens of *R. adriatica* (Group D). Our Spanish specimens show the two pads of the rachidian tooth flattened (Fig. 3B [arrow]), while in the Croatian specimens the dorsal surface of these pads are curved (Fig. 3A). Thus, here we have provided SEM micrographs of the radula and gizzard plates of a specimen collected in Croatia. The radula of *R. lucidum* sp. nov. has some differences compared with *R. adriatica*, *R. tiramisu* sp. nov. and *R. azorica* sp. nov. (see Table 3). In *R. lucidum* sp. nov. the two pads of the rachidian teeth are flattened (Fig. 3D), differing from *R. adriatica, R. tiramisu* sp. nov. and *R. azorica* sp. nov. where the pads are slightly curved on the upper part (Figs. 3A, G, J). The number of denticles also differs among *R. lucidum* sp. nov., *R. adriatica*, *R. azorica* sp. nov. and *R. tiramisu* sp. nov. (Table 3). The lateral teeth of *R. lucidum* sp. nov. are smooth like in *R. adriatica* and *R. azorica* sp. nov., but the apical cusp is longer than in the others species (Figs. 3B, E, L). *R. tiramisu* sp. nov. is the only to show the lateral teeth denticulate (Fig. 3H).

Regarding the reproductive system, the original description of *R. adriatica* only mentions that “the ovotestis contained tailed spermatozoa, but no ripe eggs” (Thompson, 1980), but there is no mention to other structures. Thompson and Brodie (1988) and Schmekel and Cappellato (2002) did not mention any details about the reproductive system. Thus, here we have completed the anatomical description of *R. adriatica* providing information about the reproductive system (see Systematic section). The female mass of *R. adriatica* has elliptical shape (Fig. 4A). In the species *R. lucidum* sp. nov., the female gland mass is similar in shape to *R. tiramisu* sp. nov. and *R. azorica* sp. nov., but the common genital duct is shorter and curved (Fig. 4B–D). While in *R. tiramisu* sp. nov. the female mass is more rounded than that in *R. azorica* sp. nov. (Fig. 4C, D). In the species *R. lucidum* sp. nov., the seminal vesicle is the thickest and longest among all species here studied, reaching more than a half size of the prostate (Fig. 5). The seminal vesicle of *R. tiramisu* sp. nov. is placed in the opposite side of the prostate compared with *R. adriatica* and *R. azorica* sp. nov. (Fig. 5 A, C, D). The atrium in *R. lucidum* sp. nov. is shorter than in *R. adriatica*, but longer than *R. tiramisu* sp. nov. and *R. azorica* sp. nov. (Fig. 5).

Concerning the specimen identified as *Runcina* sp., externally is extremely similar to *R. adriatica*, especially to our Italian specimens of this species (Fig. 2F; 8A), but lack the horizontal white bands, only showing oval white patches behind the eyes. The radula of *Runcina* sp. is also similar to that of *R. adriatica.* The rachidian tooth of *Runcina* sp. resembles that of the Spanish specimen of *R. adriatica* showing the two pads flattened (Figs 3B [arrow], 8B), but differs from the specimens of Croatia that present these pads curved (Fig. 3A). The lateral teeth are also smooth like in *R. adriatica* (Figs 3B, 8C). Despite these similarities, our species delimitation analyses clearly separate both species, but reveal a close relationship as sibling species (Fig. 1; Table 2). From the results of our analyses, we firstly thought that our species could correspond to *R. capreensis* (Mazzarelli, 1894), because *R. capreensis* is externally also very similar to *R. adriatica* and our specimen matches with the external description by Mazzarelli (1894). Thompson and Brodie (1988) discussed that using only the external features would be difficult to distinguish both species, noting that they could be conspecific. The most distinctive feature of our specimen of *Runcina* sp. and *R. adriatica* respect to *R. capreensis* is the radula. In *Runcina* sp. and *R. adriatica* the rachidian tooth is denticulate, while *R. capreensis* was described as having smooth rachidian tooth. Since only one specimen was available and it was not possible to study the reproductive system either, we decided not to assign a specific name and wait for further material to be available.

The variability in the colour pattern observed in this study (Figs 2, 6, 7, 8 and Table 3) indicates that this feature, at least in the "*R. adriatica*" species complex, is not taxonomical useful and can lead to erroneous identifications. Other external features such as the number of gills and the body shape could be useful to identify species within this complex, but they may also exhibit some variability (Thompson, 1980, Thompson and Brodie, 1988). It is important to highlight that the internal characters play a critical role to separate these species, specially the radula (Figs 3, 8B–C; Table 3), but also the reproductive system, mainly related with the shape and size of the organs (Figs 4 and 5). However, it is also necessary to mention that the fixation method could influence these differences. Regarding gizzard plates, the number of crests or laminae also varies from one species to another (Figs 3C, F, I, M and 8D; Table 3). Although internal characters provide more meaningful information than external features, anatomically studying these small heterobranch sea slugs is a great challenge.

Because the species within "*R. adriatica"* complex and most runcinids lack other external morphological characters that could help distinguish these species (cerata, rhinophores, oral tentacles, foot corners, notum tubercles, notum appendages), the colour pattern remains crucial to identify them, even being an unstable taxonomic character. Still, we emphasize the need for anatomical studies to gather as much information as possible on these species allowing a better assessment of biodiversity in Runcinida.

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Table 1. List of specimens used for phylogenetic analysis. (*) Sequences from Chapter I. ([1]) Names proposed in the Chapter I. Museum abbreviations: Museum Victoria collections (NMVF), University Museum of Bergen (ZMBN), Zoologische Staatssammlung München, Germany (ZSM), Museo Nacional de Ciencias Naturales (MNCN) and The Natural History Museum, London, United Kingdom (NHMUK).

Species	New taxonomic assignment after phylogenetic study	Locality	Voucher no.	H3	COI	16S
Ilbia ilbi Burn, 1963		Hamers Haven, Australia	NMVF234189	MK322992	-	MK323019
Lapinura divae (Ev. Marcus and Er. Marcus, 1963)		Bermuda (GB)	ZMBN 82997	KJ022893	KF992195	KJ022825
Metaruncina setoensis (Baba, 1954)		Okinawa, Japan	ZMBN 88231	-	*	*
Runcina adriatica T. Thompson, 1980	***R. azorica* sp. nov.**	Azores Islands, Portugal	Pending number	*	*	*
Runcina adriatica	***R. azorica* sp. nov.**	Azores Islands, Portugal	Pending number	*	*	*
Runcina adriatica		Ugljan Island, Croatia	MNCN 15.05/88100	MK323022	MK322967	MK322996
Runcina adriatica		Zut Island, Croatia	MNCN 15.05/88101	-	MK322968	MK322997
Runcina adriatica		Iz Island, Croatia	MNCN 15.05/88099	MK323036	MK322986	MK323015
Runcina adriatica	***R. lucidum* sp. nov.**	Ugljan Island, Croatia	Pending number	*	*	*
Runcina adriatica	***R. lucidum* sp. nov.**	Ugljan Island, Croatia	Pending number	*	*	*
Runcina adriatica	***R. lucidum* sp. nov.**	Ugljan Island, Croatia	Pending number	*	*	*

Table 1. Continued.

Species	New taxonomic assignment after phylogenetic study	Locality	Voucher no.	H3	COI	16S
Runcina adriatica		Otranto, Italy	MNCN 15.05/88102	-	MK322966	MK322993
Runcina cf. *adriatica*	***R. tiramisu* sp. nov.**	La Strea, Italy	Pending number	*	*	*
Runcina cf. *adriatica*	***R. tiramisu* sp. nov.**	Lecce, Italy	Pending number	*	*	*
Runcina cf. *adriatica*	***R. tiramisu* sp. nov.**	Lecce, Italy	Pending number	*	*	*
Runcina adriatica	***Runcina* sp.**	Otranto, Italy	Pending number	*	*	*
Runcina adriatica		Cadaqués, Spain	MNCN 15.05/88103	MK323020	MK322987	MK322994
Runcina adriatica		Palamós, Spain	MNCN 15.05/90416	*	*	-
Runcina adriatica		Cadaqués, Spain	MNCN 15.05/90417	*	*	*
Runcina adriatica		Cadaqués, Spain	MNCN 15.05/90418	*	*	*
Runcina adriatica		Cadaqués, Spain	MNCN 15.05/90419	*	*	*
Runcina africana		Tenerife, Canary Islands, Spain	NHMUK 20030791/1	KJ022942	DQ974680	KJ022780
Runcina avellana Schmeckel and Cappellato, 2001		Mataró, Spain	MNCN 15.05/88108	MK32300	MK322973	MK323028
Runcina brenkoae T. Thompson, 1980		Split, Croatia	MNCN 15.05/88086	MK322998	MK322969	MK323023

Table 1. Continued.

Species	New taxonomic assignment after phylogenetic study	Locality	Voucher no.	H3	COI	16S
Runcina brenkoae		Nin, Croatia	MNCN 15.05/88088	MK323018	MK322971	MK323039
Runcina caletae[1]		Cádiz, Spain	Pending number	*	*	*
Runcina coronata (Quatrefages, 1844)		Swanage, England	MNCN 15.05/88105	MK323010	MK322976	MK323031
Runcina ferruginea Kress, 1977		Newlyn, Cornwall, England	MNCN 15.05/88109	MK323011	MK322977	MK323032
Runcina flavonigra[1]		Cádiz, Spain	MNCN 15.05/88107	MN057639	MN057640	MN057642
Runcina hornae Schmeckel and Cappellato, 2002		Palamós, Spain	MNCN 15.05/88110	-	MK322978	MK323033
Runcina hornae		Cadaqués, Spain	MNCN 15.05/88104	MK323005	MK322988	-
Runcina cf. *hornae*	***R. lucidum* sp. nov.**	Iz Island/Parda (HR)	Pending number	*	-	*
Runcina lusitanica Araujo, Pola, Malaquias and Lucas, 2019		Faro, Portugal	MNCN 15.05/200065	MK323002	MK322981	-
Runcina lusitanica		Faro, Portugal	MNCN 15.05/90424	*	*	*
Runcina marcosi Araujo, Pola, Malaquias and Lucas, 2019		Roses, Spain	MNCN 15.05/88098	MK323000	MK322982	MK323025

Table 1. Continued.

Species	New taxonomic assignment after phylogenetic study	Locality	Voucher no.	H3	COI	16S
Runcina marcosi		Banyuls-sur-Mer, France	ZSM MOL 201442089	MK322995	MK322991	MK323021
Runcina sp.	***R. lucidum* sp. nov.**	Zut Island, Croatia	Pending number	*	*	*
Runcina sp.	***R. lucidum* sp. nov.**	Zut Island, Croatia	Pending number	*	*	*
Runcina sp.	***R. lucidum* sp. nov.**	Zut Island, Croatia	Pending number	*	*	*
Aplysia dactylomela Rang, 1828		Cape Verde (GB)	NHMUK 20030795/ 20030796	KJ022921	KF992168	KJ022798
Micromelo undatus (Bruguière, 1792)		Tenerife, Canary Island (GB)	NHMUK 20030800	KJ022944	DQ974653	KJ022778

Table 3. Differences between *Runcina adriatica*, *Runcina lucidum* sp. nov., *Runcina tiramisu* sp. nov., *Runcina azorica* sp. nov. and *Runcina* sp. Data after, Thompson (1980), Thompson and Brodie (1988), Schmekel and Cappellato (2002), Malaquias *et al*. (2009[b], 2014) and present study.

	Runcina adriatica	*Runcina lucidum* sp. nov.	*Runcina tiramisu* sp. nov.
Colour pattern	Body yellowish translucent. Dark spots on the notum, mantle groove and foot. Two white oval blotches anteriorly to eyes. Horizontal white bands behind eyes and posteriorly, on the notum edge. Longitudinal white line connects the horizontal bands. Dark oval spots on the median dorsal surface of the foot. White speckles on each side of the yellowish longitudinal band on the foot.	Body colour grey, light brown or orange-honey. Light brown specimens bear anastomosing brown blotches on notum and lateral margins of the foot. Grey specimens with brown blotches dispersed on the notum. Orange-honey specimens lack brown blotches and bear a discontinuous white line and continuous dark brown line on lateral notum edge. Chalk white spots all over body, more concentrated on posterior margin of foot and posterior end of notum. Two oval white blotches or horizontal band of white spots behind eyes. Longitudinal band of the same colour of the body on the surface of the foot.	Body brown chocolate. Notum with some lighter gaps on the lateral edges and both sides of the head. Two oval white patches behind the eyes and at the posterior end of the notum. Dorsal surface of the foot with translucent lateral edges. Median brown patch on the posterior part of the foot.
Gills	Three yellowish translucent gills with small dark and white dots.	Three small translucent gills.	Three small translucent gills with brown margins.
Shell	Absent	Absent	Absent
Radular formulae	21–33 x 1.1.1	22–25 x 1.1.1	26 x 1.1.1
Radular teeth	Rachidian tooth with two pads with upper part slightly curved or flattened with 7–8 or even 13 long denticles laminae-shaped disposed as an arc. Lateral teeth smooth, elongate and curved like a swan neck.	Rachidian tooth with two flattened pads each with 7–10 relatively long, sharped denticles laminae-shaped with the same size and disposal straight. Lateral teeth smooth, elongate and curved like a swan neck.	Rachidian tooth with two pads with upper part slightly curved each one with 8–13 long and thick denticles. Lateral teeth elongate, hooked shape with 20–25 small denticles.
Gizzard plate	Four, with 8–11 crests	Four, with 7–8 crests	Four, with 7 crests

Table 3. (Continued)

	Runcina azorica sp. nov.	*Runcina* sp.
Colour pattern	Body yellowish translucent. Dark spots on the notum, mantle groove and foot. Dark brown speckles highly concentrated on the median zone of the notum as a large dark patch. Small white spots concentrated on the median zone. Horizontal white bands behind eyes and posteriorly, on the notum edge. Fine dark orange line covers the margin of the notum. Dark brown oval spots on the median dorsal surface of the foot.	Ground colour white and yellowish translucent. Notum, mantle groove and foot covered with black dots. Small black dots concentrated at the head and posterior end of notum. Large black spots on the central zone of notum. Small white specks distributed longitudinally on the central part of the notum. Two white oval blotches behind the eyes. White bands on the lateral margins of the posterior end of the foot. Several black spots on the central zone of the dorsal surface of the posterior end of the foot.
Gills	Two yellowish translucent gills with small dark dots. Both divided into two horizontal arcs.	Two or three yellowish translucent gills with small dark spots.
Shell	Absent	Absent
Radular formulae	24–25 x 1.1.1	13 x 1.1.1
Radular teeth	Rachidian tooth with two broad and flat pads with 13–15 long denticles laminae-shaped disposed as an arc. Small denticle between pads. Lateral teeth smooth, elongated and triangular with curved tweezers shape.	Rachidian tooth with two pads slightly curved on the upper part, each with 10–11 relatively long denticles laminae-shaped with the same size disposed in arc. Lateral teeth smooth, elongated, hooked shape with a long and wide basal region.
Gizzard plate	Four, with 9 crests	Four, with 7–8 crests

SUPPLEMENTARY FIGURES

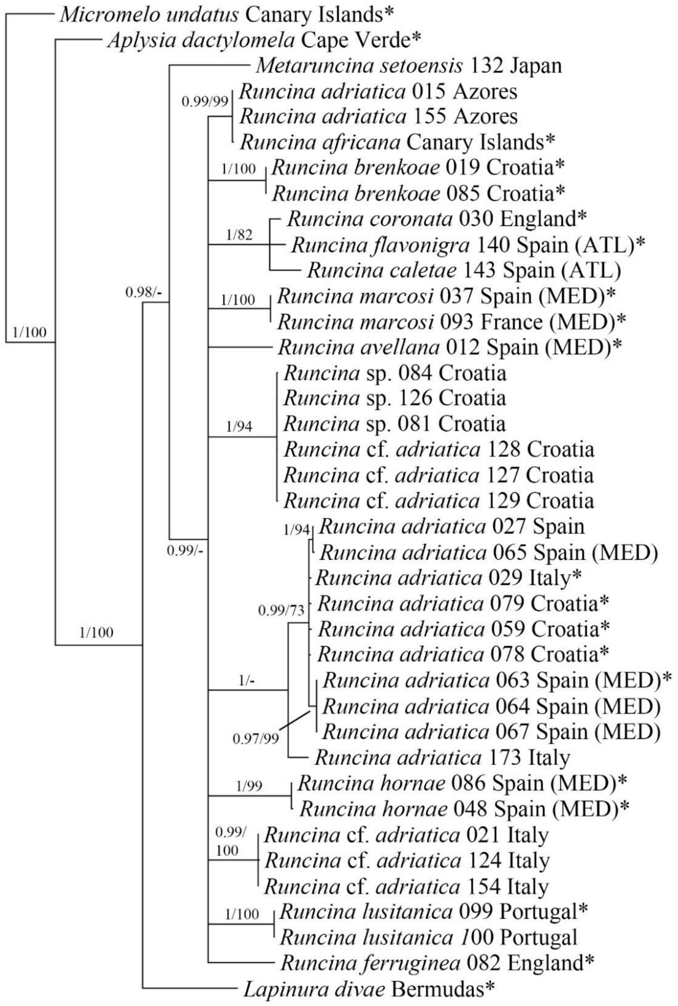

Figure S1. Phylogenetic hypothesis of "*Runcina adriatica*" complex based on BI of the COI gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

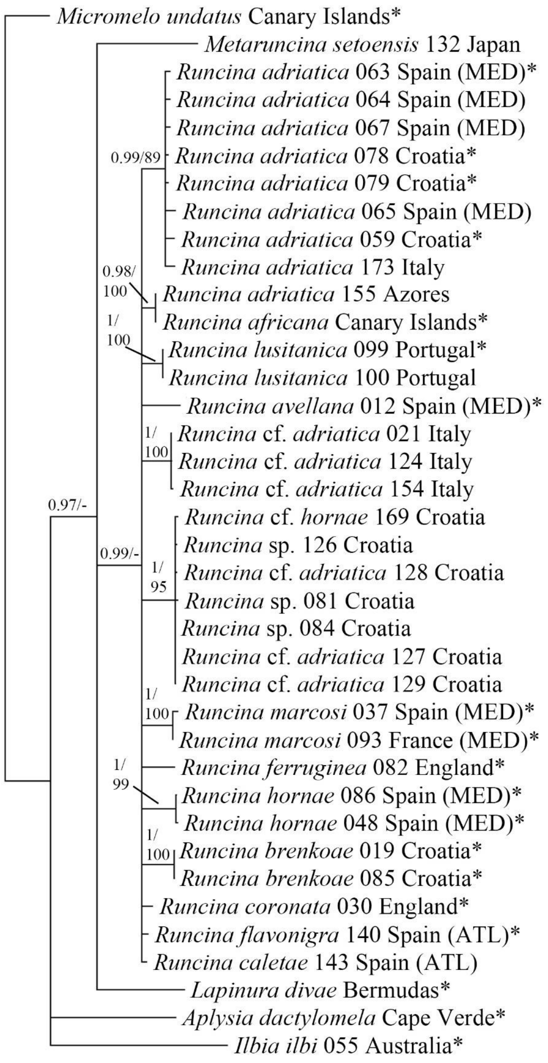

Figure S2. Phylogenetic hypothesis of "*Runcina adriatica*" complex based on BI of the 16S gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

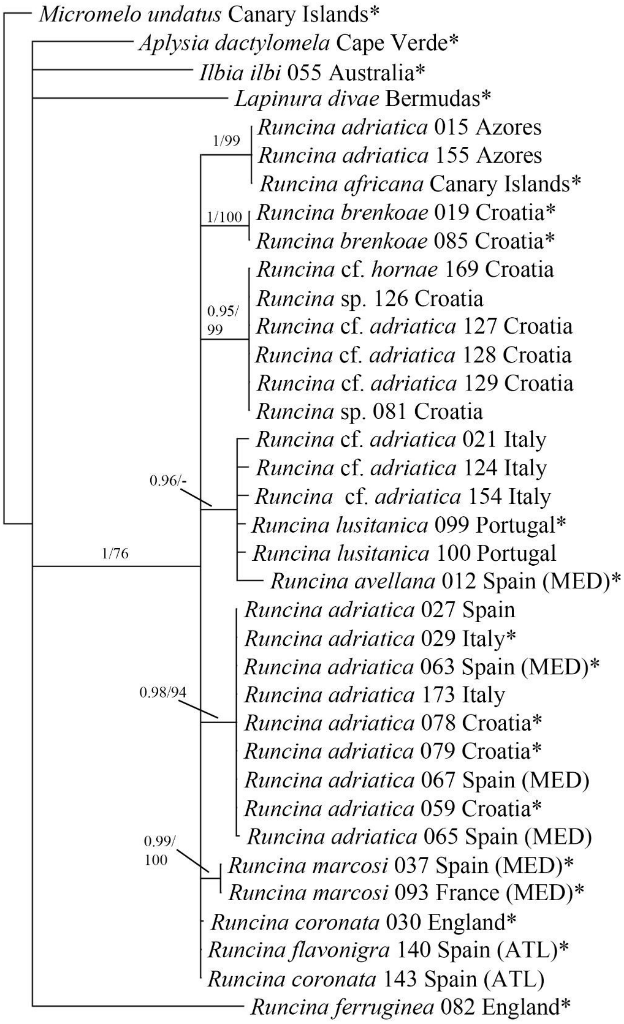

Figure S3. Phylogenetic hypothesis of "*Runcina adriatica*" complex based on BI of the H3 gene. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

DISCUSIÓN GENERAL

Hasta la fecha, la mayoría de los trabajos sobre runcínidos europeos corresponden a descripciones originales (Quatrefages, 1844, Forbes y Hanley, 1851, Thompson, 1980, Cervera *et al*., 1991, Schmekel y Cappellato, 2002, Ortea *et al*., 2013, Moro y Ortea, 2015, Ortea *et al.*, 2017, entre otros). Tras una revisión de toda la bibliografía relacionada con el orden Runcinida, es posible observar un cambio importante relativo al rango taxonómico asignado a este grupo, considerado tradicionalmente como un suborden dentro del orden Cephalaspidea (Burn, 1963, Ghiselin, 1963, Baba, 1967, Er. Marcus y Ev. Marcus, 1970, Kress, 1977, Schmekel, 1985, Thompson y Brodie, 1988, Gosliner, 1990, 1991, Cervera *et al*., 1991; Mikkelsen, 1993, 1996, Schmekel y Cappellato, 2001, 2002) y que, sin embargo, recientemente ha sido elevado al rango de Orden (Malaquias *et al*., 2009, Jörger *et al*., 2010, Göbbeler y Klussmann-Kolb, 2011, Wägele *et al*., 2014, Oskars *et al*., 2015). No obstante, en la bibliografía no existe ninguna hipótesis filogenética acerca de las relaciones entre los géneros y especies que lo integran, dado que la actual clasificación dentro del orden Runcinida está basada en caracteres morfológicos, principalmente la rádula, la branquia, las placas gástricas y la concha (Burn, 1963, Burn y Thompson, 1998). De esta forma, en la presente tesis doctoral se ha llevado a cabo el primer estudio sistemático y de relaciones filogenéticas de las especies del orden Runcinida con distribución en el Océano Atlántico europeo, incluyendo el Mar Mediterraneo. También se han incluido en el estudio algunas especies/especímenes del orden con distribución en el Atlántico Oeste y Pacifico.

Los resultados obtenidos en esta Tesis Doctoral, han contribuido al aumento del conocimiento no solamente de la biodiversidad marina europea como, también, de la biodiversidad de especies de Runcinida, que ha resultado mayor de lo que en principio se esperaba. Así, se ha revelado la existencia de cuatro complejos de especies, entre ellos el que atañe a *Runcina coronata* (Quatrefages, 1844), la especie tipo del género *Runcina*. Esto último es relevante ya que dicho género es el que abarca el mayor número de runcínidos descritos en Europa. Así, se describen un género nuevo y 10 especies nuevas para la Ciencia, además de recuperar dos nombres hasta ahora considerados como sinónimos recientes. No obstante, debido a la ausencia de más material disponible, en esta Tesis, aun han quedado especies y posibles géneros sin identificar/describir.

El problema fundamental a la hora de evaluar la diversidad taxonómica, además de intentar reconocer y delimitar especies, es el hecho de que no existe un único concepto de "especie". Hay muchos conceptos de "especie" cuyas definiciones se basan en criterios biológicos relacionados con diversas áreas de estudios como la Ecología, Genética, o Sistemática, entre otras (Tinaut y Ruano, 2003, de Queiroz, 2005, 2007). No obstante, y a pesar de las diferencias existentes entre los diferentes conceptos, existe un componente común entre todos que es la noción de “linaje” en el que, en el caso de organismos con reproducción sexual, las especies están unidas a través del proceso de flujo genético (de Queiroz, 1999, 2007, Dayrat, 2005). La problemática actual está en cuál sería el mejor criterio para reconocer esos linajes. Los defensores del “DNA barcoding” argumentan que cada especie tiene su propia secuencia diagnóstica y que, por lo tanto, las secuencias de uno o más genes pueden ser utilizadas para identificar especies (Herbet *et al*., 2003, Blaxter, 2004, entre otros). Por otro lado, la aplicación universal de ese “barcoding” y la delimitación de especies basada en distancias genéticas ha sido criticada (DeSalle *et al*., 2005, Rubinoff *et al*., 2006, Kelly *et al*., 2007, Eberhardt, 2010).

En los últimos años, la taxonomía integrativa con su enfoque multidisciplinar ha sido bastante utilizada para delimitar especies, sobre todo tratándose de complejos de especies (Dayrat, 2005). Esta multidisciplinariedad y delimitación de especies abarca múltiples perspectivas complementarias como la filogeografía, anatomía comparada, secuencias de marcadores moleculares, desarrollo embrionario, genética de poblaciones y otros. El uso de la taxonomía integrativa, en particular de filogenias moleculares, ha revelado que muchas especies de heterobranquios marinos (especialmente nudibranquios) bien caracterizados morfológicamente son, en realidad, complejos de especies (Krug *et al*., 2013, Padula *et al*., 2014, Carmona *et al*., 2015, Padula *et al*., 2016, Korshunova *et al.,* 2017, Layton *et al*., 2018, Valdés *et al*., 2018, Austin *et al*., 2018, Araujo *et al*., 2019 [ver capítulo 2], entre otros). A diferencia de otros heterobranquios marinos, la taxonomía en Runcinida es más complicada debido al reducido número de caracteres morfológicos informativos. Por lo tanto, en el presente estudio, la delimitación de especies se ha basado, inicialmente, en marcadores moleculares, lo que ha posibilitado observar la existencia de especies crípticas y pseudocrípticas dentro del orden Runcinida. Además, siempre que fue posible se estudiaron los ejemplares anatómicamente, ya que el uso de herramientas moleculares como forma de delimitar especies no invalida la importancia del uso de los datos morfológicos.

Desafortunadamente, en el caso de Runcinida, otras aproximaciones a la taxonomía integrativa como, por ejemplo, el desarrollo embrionario o la genética de poblaciones, no se han podido abordar. En primer lugar, porque la mayor parte del material estudiado proviene de museos y/o donaciones de colectores particulares, lo que implica la necesidad de una preservación previa, imposibilitando cualquier estudio desde una aproximación como las mencionadas. En segundo lugar, porque esas aproximaciones necesitan que la especie en estudio sea colectada un número mínimo de veces, lo que habitualmente no sucede, no solamente en runcínidos, sino en muchos heterobranquios en general (Megina y Cervera, 2003, Malaquias y Sprung, 2005, Megina *et al*., 2007, Vendetti *et al*., 2012 Bazzicalupo *et al*., 2018). En el caso de los runcínidos, la disponibilidad de ejemplares ha sido mucho menor que en otros grupos de animales, obteniéndose un único ejemplar por especie en algunos casos. Eso está directamente relacionado con el pequeño tamaño de estos animales los cuales no superan los 5 mm, así como la carencia de una coloración conspicua que hace que estos gasterópodos pasen generalmente desapercibidos en el medio.

Caracteres morfológicos

A pesar de que los resultados de la presente Tesis Doctoral están basados principalmente en marcadores moleculares, a la hora de describir las especies nuevas se han considerado también los caracteres morfológicos tradicionalmente utilizados en las descripciones, como son la forma y coloración del cuerpo, las branquias, la rádula y las placas gástricas. Además, también se ha detallado lo máximo posible el sistema reproductor. Los análisis de filogenia molecular llevados a cabo revelaron que estos caracteres morfológicos son, en muchos casos, poco informativos, especialmente para separar especies dentro del género *Runcina* que ha sido el taxón con el mayor número de especies aquí estudiadas. Basándonos en los resultados de la filogenia molecular y de la revisión bibliográfica, a continuación, se discute sobre la relevancia de los caracteres morfológicos aquí estudiados.

1. Forma corporal y coloración

Entre las especies del genero *Runcina*, la forma del cuerpo no presenta una variación significativa y por sí sola no es muy informativa. Algunas especies pueden presentar el perfil de la parte frontal de la región cefálica bilobulado, mientras que en otras ese perfil es rectilíneo. Asimismo, en algunas especies la región posterior del noto se extiende formando una cresta, mientras que en otras es redondeada. La longitud del cuerpo tampoco es muy informativa, pues dependiendo del estado del individuo (si está en movimiento o no) esa longitud podría variar. Incluso entre géneros este carácter no es muy útil, salvo para los géneros *Lapinura* y *Karukerina*, por ejemplo, que son los únicos con la parte posterior del noto con lóbulos y que se diferencian entre sí por la presencia de concha externa en el primero e interna en el segundo.

Respecto a la coloración, la mayoría de las especies de runcínidos tienen patrones de color crípticos oscuros, salvo especies como *Ilbia ilbi* y *Runcinella zelandica* (Fig. 1). Por ello, aunque la coloración es esencial para una identificación preliminar, los complejos de especies detectados en la presente Tesis (Capítulos I, II, III y IV) demuestran la poca fiabilidad de ese carácter, dado que un mismo patrón de color puede pertenecer a distintas especies, pero que también patrones distintos pueden corresponder a una misma especie.

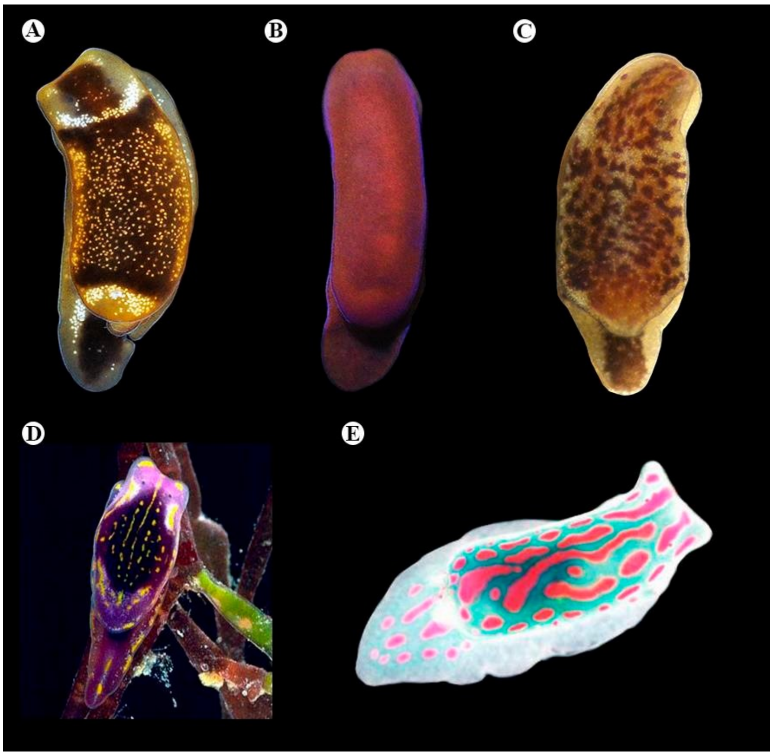

Figura 1. Ejemplos de patrones de colores del orden Runcinida. **A.** *Runcina coronata* (foto Ana Karla Araujo). **B.** *Runcina ferruginea* (foto David Fenwick). **C.** *Runcina marcosi* (foto Ana Karla Araujo). **D.** *Ilbia ilbi* (foto Bill Rudman). **E.** *Runcinella zelandica* (foto Bill Rudman).

2. Branquia/s

El número y la disposición de las hojas branquiales presentan diferencias importantes entre géneros. Burn (1963) ilustró los diferentes patrones morfológicos correspondientes a los cuatro géneros existentes hasta entonces. Tras la revisión de la bibliografía existente, en la Figura 2 se recogen los patrones existentes en el orden Runcinida, teniendo en cuenta que los géneros *Ilbia* y *Pseudoilbia* son los únicos que no presentan branquias. En base a los resultados obtenidos en esta Tesis, el carácter de la/s branquia/s ha resultado ser bastante informativo a nivel de género, aunque sólo se hayan podido analizar la mitad de los géneros conocidos. Sin embargo, a nivel de especie, este carácter es menos informativo. En el género *Runcina*, la forma y numero de hojas branquiales son muy similares entre las distintas especies (Capítulos I, II, III y IV).

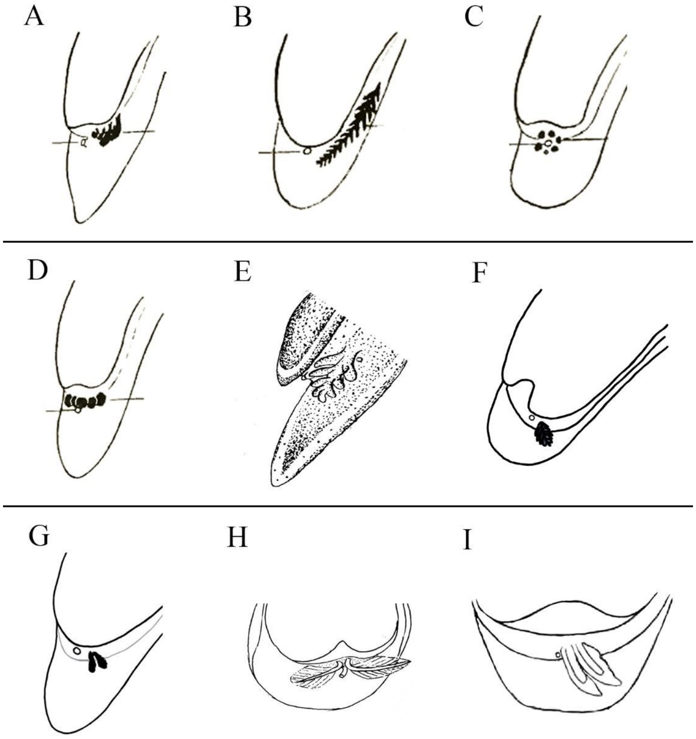

Figura 2. Disposición de las hojas branquiales en el orden Runcinida (el género *Ilbia* y *Pseudoilbia* carecen de branquias). **A.** *Runcina.* **B.** *Ildica.* **C.** *Runcinella.* **D.** *Runcinida.* **E.** *Metaruncina.* **F.** *Lapinura.* **G.** *Edmundsina* **H.** *Karukerina*. **I.** *Fofinha.* Adaptado de Burn (1963) (A–D), Baba (1967) (E), Er. Marcus y Ev. Marcus (1970) (F), Ortea (2013) (G), Ortea *et al*. (2013) (H) y Moro y Ortea (2015) (I).

3. Rádula

Dentro de las familias del orden Runcinida, la rádula presenta un aspecto bastante variable y puede ser usada para diferenciar la mayoría de los géneros que componen el orden. A partir de la bibliografía existente, en la Figura 3 se ilustran los patrones de rádula correspondientes a cada uno de los géneros. El diente raquídeo puede ser tricúspide como ocurre en el género *Ilbia* (Fig. 3E); bicúspide como en los géneros *Runcina, Runcinella, Runcinida* y

Lapinura (Fig. 3A, C, D, H); unicuspide como en *Edmundsina* y *Fofinha* (Fig. 3I, L); o incluso sin cúspide como ocurre en el género *Ildica* (Fig. 3B). En los géneros *Metaruncina, Pseudoilbia* y *Karukerina* el diente raquídeo está ausente (Fig. 3F, G, J). El género descrito en la presente Tesis, *Pseudoruncina*, es el único del orden que no presenta rádula. Los dientes laterales pueden ser lisos y/o denticulados, dependiendo del género (Fig. 3).

Sin embargo, a nivel específico, la rádula puede ser más o menos informativa y/o significativa dado que su morfología puede ser muy similar entre muchas especies. En el caso de las especies del género *Runcina* que componen los complejos de especies puestos de manifiesto en esta Tesis (ver Capítulos I, II, III y IV), las rádulas son extremadamente parecidas, siendo casi imposible diferenciar entre especies considerando solamente ese carácter, salvo algunas de las especies dentro de los complejos "*R. ferruginea*" (Cap. III) y *"R. adriatica*" (Cap. IV) que se diferencian por tener los dientes laterales denticulados, mientras que las demás los tienen lisos.

4. Placas gástricas

Entre los caracteres morfológicos utilizados para describir especies de runcínidos, las placas gástricas han mostrado ser el carácter menos informativo, dado que la mayoría de las especies poseen cuatro placas gástricas, salvo en los géneros *Pseudoilbia* y *Fofinha* que carecen de ellas. El número de crestas que posee cada placa tampoco es significativo como para separar géneros y/o especies. De esta forma, las placas gástricas solo serían significativas a nivel de orden, ya que la presencia de cuatro placas gástricas es una característica única del orden Runcinida.

5. Sistema reproductor

Aunque muchas descripciones de runcínidos no hacen mención al sistema reproductor, en las descripciones incluidas en esta Tesis se ha intentado detallar el máximo posible la información relativa al mismo con el fin de hallar características que pudiesen ayudar a distinguir una especie de otra. Los resultados obtenidos mostraron que las especies presentan diferencias en el sistema reproductor que pueden ayudar a identificarlas. Sin embargo, un mayor número de ejemplares permitiría determinar si tales diferencias son realmente significativas o no. También se hace necesario resaltar que las diferencias existentes de forma y tamaño pueden estar condicionadas por el método de fijación y tamaño del ejemplar.

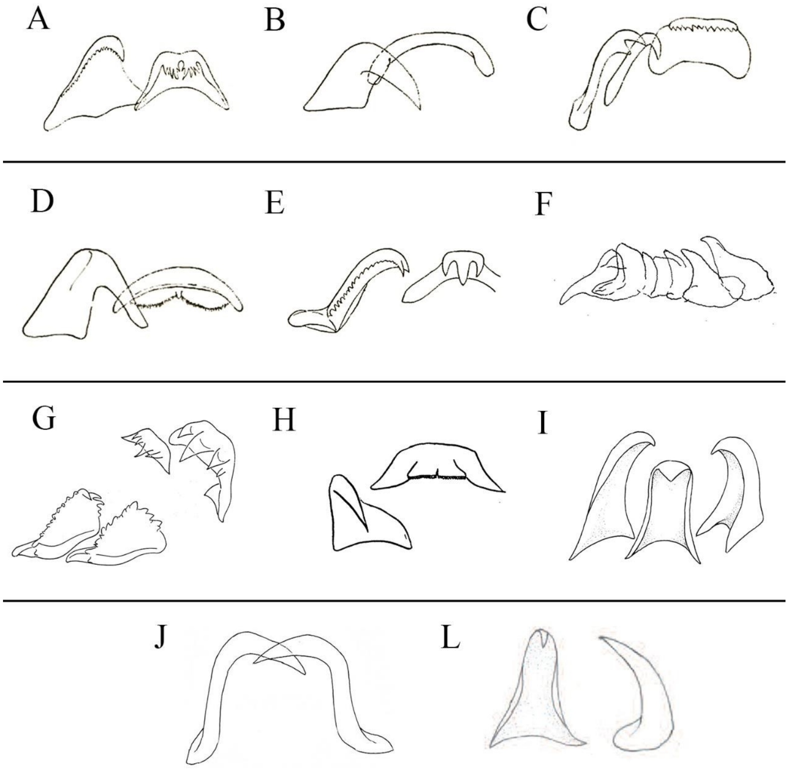

Figura 3. Diferentes morfologias de dientes radulares en Runcinida (el género *Pseudoruncina* carece de radula). **A.** *Runcina*. **B.** *Ildica.* **C.** *Runcinella.* **D.** *Runcinida.* **E.** *Ilbia* **F.** *Metaruncina* **G.** *Pseudoilbia.* **H.** *Lapinura*. **I.** *Edmundsina* **J.** *Karukerina*. **L.** *Fofinha.* Adaptado de Burn (1963) (A, B, D, E), Odhner (1924) (C), Baba (1967) (F), Miller y Rudman (1968) (G), Er. Marcus y Ev. Marcus (1970) (H), Ortea (2013) (I), Ortea *et al*. (2013) (J) y Moro y Ortea (2015) (L).

Análisis filogenéticos

Si bien el ámbito de la presente Tesis son los runcínidos europeos, la inclusión de representantes de otras regiones ha proporcionado una primera aproximación sobre las relaciones existentes dentro del orden Runcinida, con una hipótesis filogenética cuya representación gráfica presenta un elevado grado de soporte en los nodos basales. Desafortunadamente, en los análisis no se pudieron incluir todas las especies europeas de runcínidos ya que, o bien estaban fijadas en formalina o bien se carecía de material de ciertas especies. Una situación similar ha sucedido en el caso de especies de otras regiones de fuera de Europa.

Todos los análisis filogenéticos se han basado exclusivamente en secuencias parciales de nucleótidos de dos genes mitocondriales, citocromo *c* oxidasa subunidad I (COI) y 16S rRNA, y del gen nuclear histona 3 (H3). Los árboles resultantes de los análisis con los tres genes concatenados, proporcionaron una mejor resolución que aquellos basados en cada uno de los genes por separado. Además, los árboles resultantes a partir de los análisis de inferencia bayesiana (BI) y de análisis de máxima probabilidad (ML) mostraron topologías muy similares. Los resultados obtenidos tras los análisis realizados apoyaron la monofilia del orden Runcinida. No obstante, para discutir y comprender mejor el status de las familias Ilbiidae y Runcinidae se hace necesario la inclusión en los análisis filogenéticos de más taxones de ambas familias, ya que para Ilbiidae solo se ha incluido uno de sus tres géneros y para Runcinidae se han incluido cuatro de sus ocho géneros. De todos modos, las relaciones entre los géneros analizados en esta Tesis están soportadas por los análisis de inferencia bayesiana (BI), aunque los valores de bootstrap (ML) resultaron ser bajos.

Si bien únicamente se han analizados cinco de los once géneros descritos del orden Runcinida, los análisis filogenéticos no han sustentado la propuesta de Burn (1963), en la que por sus características morfológicas se consideraba el género *Runcina*, el segundo más basal tras el género *Ildica*, mientras que el género *Ilbia* se consideraba el más avanzado. Así, en todos los árboles filogenéticos del presente estudio, *Ilbia* aparece como el género más basal. No obstante, para obtener resultados más definitivos sería necesario la inclusión de representantes de los demás géneros.

A partir de los análisis filogenéticos y de la bibliografía se ha podido hacer un primer mapeo de la evolución de algunos de los caracteres aquí analizados como la concha, la/s branquia/s y la rádula. Según la hipótesis filogenética más reciente acerca de los linajes tradicionalmente denominados “opistobranquios”, los órdenes Aplysiida y Pteropoda constituyen el grupo hermano del orden Cephalaspidea, y a su vez todo este clado sería el grupo hermano de los Runcinida (Jörger *et al*., 2010, Wägele *et al*., 2014).

1. <u>Evolución de la concha</u>

Dado que la mayoría de las especies de Cephalaspidea, Aplysiida y Pteropoda poseen una concha externa (Gosliner, 1994), podemos asumir la hipótesis de que el ancestro de Runcinida también poseía una concha externa, pero extremamente reducida (Fig. 4).

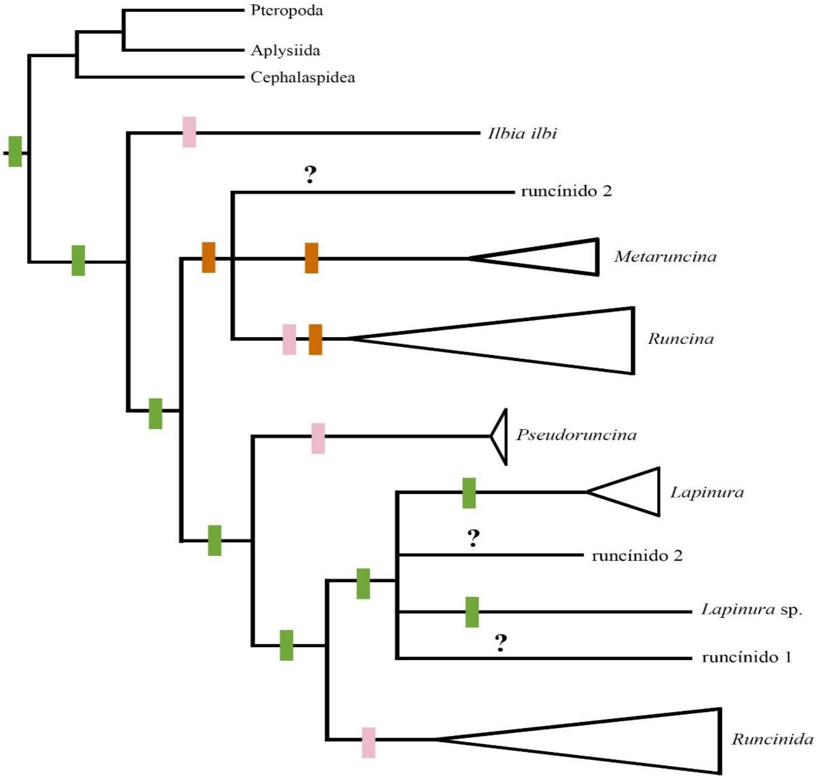

Figura 4. Hipótesis relativa a la evolución de la concha en el orden Runcinida. Leyenda: Verde, concha externa; Naranja, concha interna; Rosa, concha ausente.

En nuestros análisis, el linaje más basal representado por *Ilbia ilbi* (familia Ilbiidae) habría perdido la concha, mientras que el linaje constituido por la familia Runcinidae conservaría una concha externa reducida. Sin embargo, dentro de este linaje, algunos géneros han conservado esa concha externa reducida, como *Ildica* y *Lapinura*; otros presentan una concha interna achatada, semejante a la de muchos aplísiidos, como en los géneros *Metaruncina, Karukerina* y algunas especies de *Runcina* (*R. australis* y *R. marshae*, aunque en la especie *R. katipodes* la concha interna se presenta en forma de frijol). En las demás especies de *Runcina* y en los demás géneros la concha se ha perdido.

2. Evolución de la/s branquia/s

La branquia, en la gran mayoría de los representantes de Cephalaspidea y Aplysiida, está representa por una estructura única plisada (Gosliner, 1994). Por ello, partiendo de la hipótesis filogenética de Jörger *et al*. (2010) y Wägele *et al*., (2014) podemos asumir que el ancestro de Runcinida también poseía una única branquia plisada, situada posteriormente en el lado derecho del cuerpo debido a una detorsión completa de la masa visceral. Según nuestra hipótesis filogenética para Runcinida (Fig. 5), el linaje más basal (*Ilbia ilbi*, familia Ilbiidae) habría perdido completamente la branquia. Sin embargo, el linaje que representa la familia Runcinidae conservaría esa branquia única plisada en el lado derecho del cuerpo. No obstante, a lo largo de la evolución la familia habría sufrido varios cambios en el número y la posición de la/s branquia/s.

El clado de la familia Runcinidae está compuesto por dos subclados. En el primero (Fig. 5, barra descontinua), el ancestro también habría presentado una branquia única y plisada en el lado derecho, la cual se conservaría en el género *Metaruncina*. Sin embargo, en el género *Runcina* esa única branquia habría pasado a un número de 2–4, dependiendo de la especie. En el segundo subclado (Fig. 5, barra continua), se propone la hipótesis del cambio de una única branquia a dos branquias plisadas en el ancestro del mismo. Ese tipo de branquia se conservaría en el género descrito en esta Tesis, *Pseudoruncina*. Sin embargo, en el linaje que habría dado origen a los géneros *Lapinura* y *Runcinida*, habría surgido un nuevo cambio en el número de branquias, de dos a cinco, y en su posición, formando un semicírculo por encima del ano. Ese tipo de branquia se ha mantenido en el género *Runcinida*, pero en *Lapinura* hubo una reducción posterior de cinco a tan solo una branquia situada en el lado derecho del ano. La Figura 6 plantea la hipotética evolución de la branquia en este subclado.

3. Evolución de la rádula

Por lo general, en los grupos integrantes del clado hermano de Runcinida (ver Figs 4 y 5), la rádula es del tipo multiseriado con diente raquídeo (Gosliner, 1994). No obstante, en algunos miembros de estos grupos se puede observar la reducción del número de dientes laterales, la pérdida del diente raquídeo o incluso la ausencia total de la rádula. En el caso de Runcinida, la rádula es triseriada (1.R.1) en la gran mayoría de sus miembros (Thompson y Brodie, 1988). Sin embargo, en el género *Runcinella* se observa una rádula con dientes lateralesa ambos lados del diente raquídeo (2.R.2). En otros géneros, la rádula presenta uno o dos dientes laterales por hemifila, aunque el diente raquídeo está ausente como en *Pseudoilbia* (2.0.2) y *Metaruncina* (1.0.1). El género *Pseudoruncina*, descrito en la presente Tesis, representa el primer caso de ausencia de esta estructura en Runcinida.

En la literatura, la disposición 1.R.1 de la rádula en Runcinida es considerada como una situación primitiva (Gosliner, 1994). Desafortunadamente, en nuestros análisis, no se ha podido plantear ninguna hipótesis de cómo sería la disposición de rádula en el ancestro, ya que no se han podido incluir representantes de los géneros *Runcinella* y *Pseudoilbia*.

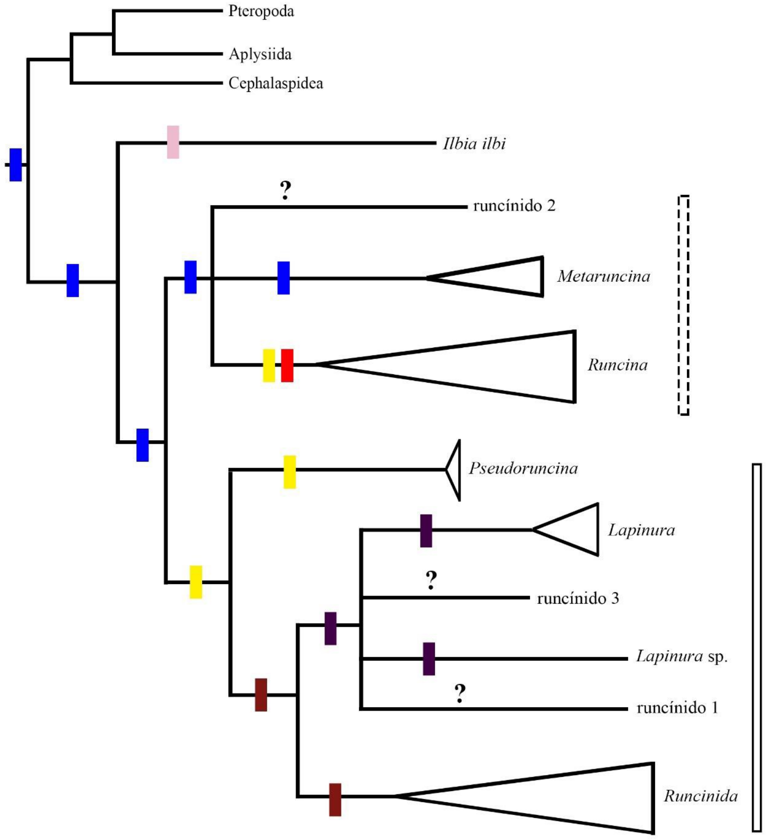

Figura 5. Hipótesis relativa a la evolución de la branquia en el orden Runcinida. Leyenda: Azul, una branquia del lado derecho del ano; Rojo, más de dos branquias del lado derecho del ano; Amarillo, dos branquias del lado derecho del ano; Marrón, cinco branquias dispuestas en semicírculo arriba del ano; Morado, una branquia del lado derecho del ano; Rosa, branquia ausente.

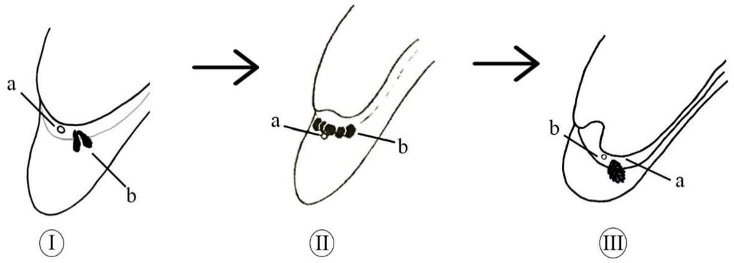

Figura 6. Hipótesis de la posible evolución de la branquia en el subclado formado por *Pseudoruncina* (I), *Runcinida* (II) y *Lapinura* (II) de la figura 5. No hay información para los otros dos linajes dentro del subclado. Leyenda: a, ano; b, branquia. Ilustraciones I y III de Ana Karla Araujo. Ilustracion II adaptado de Burn (1963).

Tareas para el futuro

A través del presente estudio se ha ampliado el conocimiento sobre la biodiversidad de Runcinida en Europa. Además, al incluir material de otras regiones del mundo, se han comenzado a comprender las relaciones filogenéticas entre los miembros de ese orden. Sin embargo, el estudio del orden Runcinida tanto en Europa como en el mundo no está completo.

En el futuro, será necesario llevar a cabo nuevos muestreos en sitios clave como localidades tipos y/o áreas cercanas para intentar localizar aquellas especies de las que se carece material para estudios moleculares y/o morfológicos. Un ejemplo de sitio clave son las Islas Canarias, en donde se concentran casi mitad de las especies presente en Europa y a cuyas especies no se tuvo acceso. También sería importante colectar material de muchos linajes (géneros *Ildica, Pseudoilbia, Runcinella, Edmundsina, Karukerina* y *Fofinha*) que no pudieron ser incluidos en los análisis llevados a cabo, así como material adicional del género *Ilbia* para intentar amplificar el gen mitocondrial COI. Es cierto que existe una discrepancia entre el número de especies de runcínidos presentes en Europa y en el resto del mundo, pero seguramente eso se debe a un esfuerzo de muestro muy bajo o inexistente en las demás regiones del mundo.

Finalmente, se seguirá llevando a cabo el estudio de los runcínidos en Europa con material adicional obtenido tras la finalización de los análisis presentados en esta Memoria. Además, también, se seguirán haciendo intentos de amplificar y secuenciar los genes que faltan para algunos especímenes analizados con el fin de incrementar los soportes y resolver las relaciones entre algunos linajes.

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CONCLUSIONES

1. Los resultados de nuestros análisis filogenéticos apoyan la monofilia del orden Runcinida.
2. Nuestros anasilis filogenéticos sitúan al género *Ilbia* en una posición más basal respecto a los genéros *Metaruncina, Runcina* y *Lapinura*.
3. Los datos moleculares corroboran las diferencias morfológicas existentes entre los géneros *Runcina* y *Lapinura*. Asimismo, se retoma la denominación original de *Lapinura divae*, frente a su inclusión en el género *Runcina.*
4. Por primera vez se ha detectado la presencia del género *Runcinida* en aguas europeas. Hasta la fecha, este género se consideraba restringido al océano Pacifico (Rusia y Japón). El único espécimen encontrado presentó diferencias morfológicas y moleculares respecto a las tres especies descritas hasta la fecha (*R. elioti, R. marisae* y *R. valentinae*).
5. Nuestros análisis filogenéticos rechazan *Pseudoilbia avellana* como nombre valido. De este modo, se rescata el nombre original de la especie, *Runcina avellana.*
6. El género *Runcina* se mostró mucho más diverso de lo que se creía en un principio.
7. Se confirma la monolifia del género *Runcina*, una vez excluido algunos especímenes inicialmente identificados como *R. ferruginea* (noreste de España, Francia [Mediterráneo] y Croatia). Dentro de este género se ha detectado la existencia de cuatro complejos de especies ("*R. coronata*", "*R. brenkoae*", "*R. ferruginea*" y *R. adriatica*").
8. *Runcina hornae* es una especie con un patrón de coloración notablemente diverso, incluyendo especímenes muy similares al de la especie *R. bahiensis*.
9. *Runcina coronata*, la especie tipo del género, es un complejo de especies. Así, se describen tres especies nuevas para la ciencia: *R. flavonigra, R. caletae* y *R. tingitana*. Las dos primeras especies son simpátricas.
10. *Runcina brenkoae* también resultó ser un complejo de tres especies. Se redescribe *R. brenkoae*, así como se describen dos especies nuevas para la ciencia: *R. marcosi* y *R. lusitanica*.
11. Los datos moleculares muestran la existencia de un complejo de cuatro especies bajo el nombre de *Runcina ferruginea*. Así, se redescribe *R. ferruginea* y el nombre de *R. zavodniki* es recuperado para los ejemplares castaño-rojizos/rojizos de las costas de Croatia. Por otro lado, se describe la especie *R. lupiaensis* para aquellos runcínidos castaño-rojizos del sur de Italia, así como se propone un género nuevo, *Pseudoruncina*, a partir de ejemplares rojizos de runcínidos colectados en las costas mediterráneas españolas y francesas. La especie tipo de este género, *P. marinae*, también es descrita como nueva para la Ciencia en la presente Tesis.

12. La filogenia molecular ha revelado *R. adriatica* como un complejo de cinco especies. Se describen tres especies nuevas: *R. lucidum, R. tiramisu* y *R. azorica*. A la especie restante no se le asigna ningún nombre especifico (*Runcina* sp.), a la espera de material adicional, ya que solo se disponía de un único espécimen.
13. Los caracteres morfológicos tradicionalmente utilizados en las descripciones (forma corporal, coloración, branquias, rádula y placas gástricas) resultaron ser por si solos muy poco informativos y de poca fiabilidad a la hora de separar especies. Por otro lado, para diferenciar géneros eses caracteres son algo más significativos. El sistema reproductor, tampoco, es un carácter significativo dado que las diferencias existentes tanto en la forma como tamaño pueden estar influenciadas por el método de fijación y tamaño del ejemplar.
14. El estudio realizado en la presente Tesis Doctoral ha puesto de manifesto la necesidad de produdizar en elos estudios basados en marcadores moleculares de especies cuya distribución reconocida incluya tanto localidades antlánticas como mediterráneas.

ANEXO I

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40 Años 1979 · 2019 Universidad con futuro
cei·mar eide·mar

A QUIEN PUEDA INTERESAR,

Dña. Marta Pola Perez, D. Manuel Antonio E. Malaquias y D. Juan Lucas Cervera Currado, como coautores del artículo de investigación titulado:

To be or not to be? What molecules say about *Runcina brenkoae* Thompson, 1980 (Gastropoda: Heterobranchia: Runcinida)
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MANIFESTAMOS nuestra conformidad para la presentación de dicho artículo como parte de esta tesis doctoral, perteneciente al PD en Gestión y Conservación del Mar (EIDEMAR-UCA) por la doctoranda Ana Karla Araujo Moreira, renunciando a presentarlo como parte de otra tesis doctoral en cualquier otra Universidad.

En Cádiz, a 03 de febrero de 2020 .

Manuel António [illegible] Malaquias
Fdo.:

Marta.P
Fdo.:
Fdo.: Juan Lucas Cervera Currado
Fdo.:

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To be or not to be? What molecules say about *Runcina brenkoae* Thompson, 1980 (Gastropoda: Heterobranchia: Runcinida)

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Summary: Runcinids are poorly known minute marine slugs inhabiting intertidal and shallow subtidal rocky shores. Among the European species, *Runcina brenkoae*, described from the Adriatic Sea in the Mediterranean, has been described to display chromatic variability, placing in question the true identity and geographic distribution of the species. In this paper we investigate the taxonomic status of *R. brenkoae* based on specimens from the central and western Mediterranean Sea and the southern Iberian coastline of Portugal and Spain, following an integrative approach combining multi-locus molecular phylogenetics based on the mitochondrial markers cytochrome *c* oxidase subunit I and 16S rRNA and the nuclear gene histone H3, together with the study of morpho-anatomical characters investigated by scanning electron microscopy. To aid in species delimitation, the Automatic Barcode Gap Discovery and Bayesian Poisson tree process methods were employed. Our results indicate the existence of a complex of three species previously identified as *R. brenkoae*, namely two new species here described (*R. marcosi* n. sp. and *R. lusitanica* n. sp.) and *R. brenkoae* proper.

Keywords: Runcinida; DNA barcoding; species delimitation; integrative taxonomy; biodiversity; phylogeny.

¿Ser o no ser? Que dicen las moléculas sobre *Runcina brenkoae* Thompson, 1980 (Gastropoda: Heterobranchi: Runcinida)

Resumen: Los runcináceos son pequeñas babosas marinas poco conocidas que habitan en costas rocosas intermareales y submareales poco profundas. Entre las especies europeas, *Runcina brenkoae* descrita originalmente en el mar Adriático en el Mediterráneo, se describió mostrando una variabilidad cromática que cuestionaba la verdadera identidad de la especie y su distribución geográfica. En este artículo, investigamos el estatus taxonómico de *R. brenkoae* a partir de especímenes del mar Mediterráneo central y occidental, y del sur de las costas Ibéricas de Portugal y España, siguiendo un enfoque integrador que combina una filogenia molecular multi-locus basada en los marcadores mitocondriales citocromo c oxidasa subunidad I y 16S rRNA, y el gen nuclear histona H3, junto con el estudio de los caracteres morfoanatómicos estudiados mediante microscopía electrónica de barrido. Para ayudar en el proceso de delimitaciones de especies, se emplearon los métodos "Automatic Barcode Gap Discovery" y el "Bayesian Poisson Tree Processes". Nuestros resultados ponen de manifiesto la existencia de un complejo de tres especies previamente identificadas como *R. brenkoae*, a saber, dos nuevas especies aquí descritas (*R. marcosi* n. sp. y *R. lusitanica* n. sp.) y *R. brenkoae* propiamente dicha.

Palabras clave: Runcinida; DNA barcoding; delimitación de especies; taxonomía integrativa; biodiversidad; filogenia.

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INTRODUCTION

Runcinids are small heterobranch sea slugs with an average size of about 4 mm. The largest species known is *Runcinida elioti* (Baba, 1937) from Amakusa (Japan) which reaches a maximum length of 8 mm (Burn 1963). These slugs inhabit intertidal and shallow rocky shores and are specialized herbivores, feeding on macrophytic algae (Burn 1963, Thompson and Brodie 1988, Schmekel and Cappellato 2001). They are characterized by having an undivided dorsum, a foot lacking parapodial lobes, and an anus located next to the gill under the right posterior side of the mantle. An external or internal vestigial shell may be present, but it is absent in most species (Thompson 1976, Burn and Thompson 1998, Schmekel and Cappellato 2001).

The runcinids have traditionally been included in the order Cephalaspidea based on anatomical features such as nervous and reproductive systems (Ghiselin 1963, Kress 1977, Schmekel 1985). However, Malaquias et al. (2009), based on molecular phylogenetic analyses, demonstrated that runcinids were not part of the Cephalaspidea radiation but warrant their own ordinal assignment, a suggestion first proposed by Odhner (1968) and later corroborated by Jörger et al. (2010), Wägele et al. (2014) and Oskars et al. (2015).

The order Runcinida (Burn 1963) comprises two families, Runcinidae H. Adams and A. Adams, 1854 and Ilbiidae Burn, 1963 with nine and two genera, respectively. Within the family Runcinidae, *Runcina* is the most species-rich genus, with 38 valid species, of which 29 occur in European waters (Cervera et al. 2004, Schmekel and Cappellato 2002, Ortea et al. 2015). The small size of these animals and the fact that most species have dark, dull cryptic colour patterns render the runcinids difficult to detect and identify.

One of the taxonomically difficult species of the European fauna is *Runcina brenkoae*, Thompson, 1980, which, together with *Runcina adriatica* Thompson, 1980 and *Runcina zavodniki* Thompson, 1980, has been described from the Adriatic Sea. *Runcina brenkoae* is characterized by an elongated body with a characteristic pattern of anastomosing black blotches, a red-brown ground colour, clusters of chalk-white spots on both sides of the head behind the eyes, and presence of two gills. However, Thompson and Brodie (1988) referred to specimens of *R. brenkoae* collected near Rovinj (Croatia), the type locality, which depicted several differences in respect to the original description: the presence of a developed crest, a pale fawn ground colour and the absence of white spots. Nevertheless, the specimens possessed key features of the species: the anastomosing black blotches and presence of only two gills. Schmekel and Cappellato (2002) reported the species outside the Adriatic Sea for the first time in Banuyls-sur-Mer (French Mediterranean coast) and Ballesteros et al. (2016) reported *R. brenkoae* in Catalonia (Spanish northeastern coast).

The use of integrative taxonomic approaches, and in particular of molecular phylogenetics, has revealed the existence of numerous species complexes and contributed to the discovery of unknown species among heterobranch sea slugs (Padula et al. 2014, Austin et al. 2018, Krug et al. 2018, among others). The variable chromatic patterns described for *R. brenkoae* hint at yet another possible example of cryptic diversity masked under a single species name, but to date the taxonomy of this elusive species has only been studied on the basis of morphology.

Here we investigate for the first time the taxonomic status of the taxonomically difficult species *Runcina brenkoae* following an integrative approach combining multi-locus molecular phylogenetics and morpho-anatomical characters, based on specimens from the central and western Mediterranean Sea and the southern Iberian coastline of Portugal and Spain.

MATERIALS AND METHODS

Taxon sampling

Specimens identified as *Runcina brenkoae* were collected by the authors and colleagues from algae and seagrass or were obtained on loan from the Zoologische Staatssammlung München, Germany (ZSM). Specimens were photographed alive and preserved in 96% EtOH. The newly collected material was deposited at the Museo Nacional de Ciencias Naturales (MNCN), Madrid, Spain.

For the molecular analyses we also obtained sequences of *Ilbia ilbi* Burn, 1963 and additional *Runcina* species, namely *R. adriatica* Thompson, 1980, *R. ferruginea* Kress, 1977, *R. hornae* Schmekel and Cappellato, 2001 and *R. coronata* (Quatrefages, 1844), plus two specimens previously identified as *Runcina* cf. *bahiensis* Cervera, Garcia-Gomez and Garcia, 1991 and *Runcina* cf. *hansbechi* Schmekel and Cappellato, 2001. Furthermore, sequences of the runcinid *Lapinura divae* (Ev. Marcus and Er. Marcus, 1963), the acteonoid *Micromelo undatus* (Bruguière, 1792) and the aplysiid *Aplysia dactylomela* Rang, 1828 were obtained from GenBank and included in the analyses (Table 1).

DNA extraction, amplification and sequencing

Tissue samples were taken from the foot and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Partial sequences of the mitochondrial *cytochrome c oxidase subunit I* (COI), and *16S rRNA*(16S), and nuclear *histone H3* (H3) genes were amplified by polymerase chain reaction (PCR) using the universal primers LCO1490 and HCO2198 (Folmer et al. 1994 for COI); 16S ar-L and 16S br-H (Palumbi et al.1991 for 16S); and H3aF and H3aR (Colgan et al. 1998 for H3). PCRs were conducted in a 25 µl reaction volume containing 1 µl of both forward and reverse primers (10 µM), 2.5 µl of dNTP (2 mM), a gene-dependent amount of magnesium chloride (25 mM), 0.25 µl of Qiagen DNA polymerase (5 units/µl), 5 µl of "Q-solution" (5x), 2.5 µl of Qiagen buffer (10x) (Qiagen Taq PCR Core Kit) and 2 µl of genomic DNA. Amplification of COI was performed with an initial denaturation for 5 min at 94°C, followed by 35-36 cycles

Table 1. – List of specimens used for phylogenetic analysis. (*) New sequences generated for this study. GB: GenBank. Museum abbreviations: Museum Victoria collections (NMVF), University Museum of Bergen (ZMBN) Zoologische Staatssammlung München, Germany (ZSM), Museo Nacional de Ciencias Naturales (MNCN) and The Natural History Museum, London, United Kingdom (NHMUK).

Species	New taxonomic assignment after phylogenetic study	Locality	Voucher no.	H3	COI	16S
Ilbia ilbi Burn, 1963		Hamers Haven, Australia	NMVF234189	MK322992*	-	MK323019*
Lapinura divae (Ev. Marcus and Er. Marcus, 1963)		Bermuda (GB)	ZMBN 82997	KJ022893	KF992195	KJ022825
Runcina sp. 1	*Runcina adriatica* T. Thompson, 1980	Iz Island, Croatia	MNCN 15.05/88099	MK323015*	MK322986*	MK323036*
Runcina adriatica T. Thompson, 1980		Ugljan Island, Croatia	MNCN 15.05/88100	MK322996*	MK322967*	MK323022*
Runcina adriatica		Zut Island, Croatia	MNCN 15.05/88101	MK322997*	MK322968*	-
Runcina adriatica		Otranto, Italy	MNCN 15.05/88102	MK322993*	MK322966*	-
Runcina adriatica		Cadaqués, Spain	MNCN 15.05/88103	MK322994*	MK322987*	MK323020*
Runcina adriatica	*Runcina marcosi* n. sp.	Banyuls-sur-Mer, France	ZSM MOL 201442089	MK322995*	MK322991*	MK323021*
Runcina brenkoae T. Thompson, 1980		Split, Croatia	MNCN 15.05/88086	MK322998*	MK322969*	MK323023*
Runcina sp.4	*Runcina brenkoae*	Nin, Croatia	MNCN 15.05/88089	MK323017*	MK322972*	MK323038*
Runcina sp.5	*Runcina brenkoae*	Nin, Croatia	MNCN 15.05/88088	MK323018*	MK322971*	MK323039*
Runcina brenkoae		Roses, Spain	MNCN 15.05/88087	MK323001*	MK322970*	MK323026*
Runcina brenkoae		Roses, Spain	MNCN 15.05/88090	MK323003*	MK322964*	-
Runcina brenkoae	*Runcina marcosi* n. sp.	Roses, Spain	MNCN 15.05/88098	MK323000*	MK322982*	MK323025*
Runcina sp.3	*Runcina marcosi* n. sp.	Mataró, Spain	MNCN 15.05/88095	MK323016*	MK322983*	MK323037*
Runcina cf. sp.4	*Runcina marcosi* n. sp.	Roses, Spain	MNCN 15.05/88097	MK323012*	MK322990*	-
Runcina sp.14	*Runcina marcosi* n. sp.	Roses, Spain	MNCN 15.05/88096	MK323013*	MK322984*	MK323034*
Runcina cf. *brenkoae*	*Runcina marcosi* n. sp.	Cádiz, Spain	MNCN 15.05/200066	MK323006*	MK322985*	-
Runcina brenkoae	*Runcina lusitanica* n. sp.	Faro, Portugal	MNCN 15.05/88091	MK322999*	MK322979*	MK323024*
Runcina brenkoae	*Runcina lusitanica* n. sp.	Faro, Portugal	MNCN 15.05/200065	MK323002*	MK322981*	-
Runcina brenkoae	*Runcina lusitanica* n. sp.	Faro, Portugal	MNCN 15.05/88093	MK323004*	MK322965*	-
Runcina brenkoae	*Runcina lusitanica* n. sp.	Faro, Portugal	MNCN 15.05/88092	MK323014*	MK322980*	MK323035*
Runcina brenkoae	*Runcina lusitanica* n. sp.	Faro, Portugal	MNCN 15.05/88094	-	MK322989*	MK323027*
Runcina cf. *bahiensis* Cervera, Garcia-Gomez and Garcia, 1991		Cadaqués, Spain	MNCN 15.05/88104	MK323005*	MK322988*	-
Runcina coronata (Quatrefages, 1844)		Swanage, England	MNCN 15.05/88105	MK323010*	MK322976*	MK323031*
Runcina coronata		Cádiz, Spain	MNCN 15.05/88106	MN057641*	-	MN057638*
Runcina coronata		Cádiz, Spain	MNCN 15.05/88107	MN057642*	MN057640*	MN057639*
Runcina cf. *hansbechi* Schmeckel and Cappellato, 2001		Mataró, Spain	MNCN 15.05/88108	MK32300*	MK322973*	MK323028*
Runcina ferruginea Kress, 1977		Newlyn, Cornwall, England	MNCN 15.05/88109	MK323011*	MK322977*	MK323032*
Runcina hornae Schmeckel and Cappellato, 2002		Palamós, Spain	MNCN 15.05/88110	-	MK322978*	MK323033*
Aplysia dactylomela Rang, 1828		Cape Verde (GB)	NHMUK 20030795/ 20030796	KJ022921	KF992168	KJ022798
Micromelo undatus (Bruguière, 1792)		Tenerife, Canary Island (GB)	NHMUK 20030800	KJ022944	DQ974653	KJ022778

of 1 min at 94°C, 30s at 45°C (annealing temperature) and 1 min at 72°C, with a final extension of 10 min at 72°C. Amplification of 16S began with an initial denaturation for 5 min at 94°C, followed by 35-36 cycles of 1 min at 94ºC, 30s at 42 and 49°C (annealing temperatures) and 1 min at 72°C, with a final extension of 10 min at 72°C. Amplification of H3 was performed with an initial denaturation for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 30s at 52°C (annealing temperature) and 1 min at 72°C, with a final extension of 10 min at 72°C. Successful PCR products were sent to Macrogen, Inc for purification and sequencing on a 3730XL DNA sequencer (Applied Biosystems). All new DNA sequences have been deposited in GenBank (Table 1).

Phylogenetic analyses

Sequences were edited in Genious v10.2.3 (Drummond et al. 2009) and aligned using MAFFT (Katoh et al. 2009) implemented in Geneious v10.2.3 (Drummond et al. 2009) with the default settings (Auto [FFT-NS-1, FFT-NS-2, FFT-NS-i or L-INS-i; depends on data size]). Sequences from the protein-coding genes COI and H3 were translated into amino acids to check for stop-codons. Hypervariable regions of the 16S alignment where homology could not be confidently established were removed using Gblocks under relaxed settings (Talavera and Castresana 2007). Nevertheless, analyses including and excluding these regions provided similar results. Therefore, final analyses were performed including all bases. Sequences of the COI, 16S and H3 genes were trimmed to 658, 457 and 328 nucleotides, respectively. All three genes were concatenated using Mesquite v3.2 (Maddison and Maddison 2018), resulting in a final dataset of 1443 base pairs. Single gene and concatenated (H3+COI+16S) analyses were performed. Saturation for the first, second and third codon positions of the COI and H3 genes were calculated in MEGA v7.0 (Kumar et al. 2016).

The best-fit evolutionary model for each gene was determined in jModeltest v2.1.6 (Guindon and Gascuel 2003, Darriba et al. 2012) under the Akaike information criterion (Akaike 1974). The GTR + G + I model was selected for the COI and H3 genes, and GTR + G for the 16S gene. Bayesian inference (BI) analyses were performed in MrBayes v. 3.2.1 (Ronquist and Huelsenbeck 2003) with a random starting tree and two parallel runs of 10^7 generations. Convergence was checked in TRACER v1.7.1 (Rambaut et al. 2018) with a burn-in of 25%. Nodes with a posterior probability (PP) ≥ 0.95 (Alfaro et al. 2003) were considered well supported and discussed. Maximum likelihood (ML) analysis was executed using RAxML v8 (Stamatakis 2014) and node support was assessed with nonparametric bootstrapping (BS) with 5000 replicates. Nodes with bootstrap values (BS)$\geq$70 (Hillis and Bull 1993) were considered significant and were discussed. Both BI and ML trees were visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Minimum and maximum pairwise uncorrected *p*-distances of COI within and between species were calculated in MEGA v7.0 using all sequences available. (Kumar et al. 2016).

Species delimitation analyses

The Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012) and Bayesian Poisson tree processes (bPTP) (Zhang et al. 2013) were used to aid delimitation of species. For the ABGD we used the alignment from the fast-evolving COI gene with default settings (P_{min}=0.001, P_{max}=0.1, Steps=10, X=1.2, Nb bins=20) under the three models of evolution available, namely Jukes-Cantor (JC69), Kimura (K80) and Simple Distance. The bPTP analysis is an updated version of the original maximum likelihood PTP (modelling speciation in terms of the number of substitutions), which adds Bayesian support values to delimit species. The bPTP analyses were run with the COI and 16S trees using the webserver (https://species.h-its.org/ptp/) (Zhang et al. 2013).

Morphology

To complete and compare the results obtained by molecular phylogenetics and species delimitation analyses, specimens previously identified as *Runcina brenkoae* and *Runcina* sp. from Croatia (Adriatic) (3), Catalonia (Mediterranean, Spain) (6), Cádiz (Atlantic, Spain) (1) and Algarve (Atlantic, Portugal) (5), and one specimen early identified as *R. adriatica* from Banyuls-sur-Mer (France) were studied for their morpho-anatomy. Animals were dorsally dissected and the buccal bulbs were extracted and dissolved in a solution of 10% sodium hydroxide to expose the radula. The radulae and gizzard plates were then immersed in water, dried and mounted for scanning electron microscopy (SEM) with a Nova NanoSEM 450 available at the University of Cádiz (Cádiz, Spain). The reproductive system was examined and drawn using a dissecting microscope with the aid of a camera lucida.

RESULTS

Phylogenetic analyses

The concatenated (H3+COI+16S) tree provided better resolution than the individual gene analyses (Fig. 1, and Supplementary material Figs S1, S2 and S3). No saturation was observed, even in the third codon position. Both BI and ML analyses supported the monophyly of the genus *Runcina* (PP=1; BS=100) and showed *L. divae* to be its sister lineage (PP=0.98; BS=86). The species *Ilbia ilbi* was rendered sister to the *Lapinura* + *Runcina* clade (PP=1; BS=100). In the *Runcina* clade the species *R. ferruginea* was rendered sister to a sub-clade containing all remaining species (PP=1; BS=80). The specimens previously identified as *R. brenkoae* split into three subclades all with maximum support (PP=1; BS=100). The first clade (Group A) includes specimens from Portugal; the second clade (Group B) includes one specimen previously identified as *Runcina adriatica* from France (Mediterranean) and specimens from Spain (Atlantic and Mediterranean); and the third clade (Group C) includes specimens from Croatia and Spain (Mediterranean) (Fig. 1).

Species delimitation analyses

The ABGD analysis of the COI sequences with all three models of evolution resulted in 11 groups with three of them corresponding to the same *R. brenkoae* groups, A, B and C, recovered in the BI and ML analyses (Fig 1A). However, the recursive partition, at lower values of prior intraspecific divergence (P), recovered seven groups for the "*R. brenkoae* complex", separating specimens from Group A and C into two distinct groups each, and specimens from Group B into three distinct groups (not shown).

Regarding the COI uncorrected *p*-distances, the minimum distance was 11.7% between Groups A and B; 9.6% between Groups A and C; and 10.4% between Groups B and C. The maximum distance was

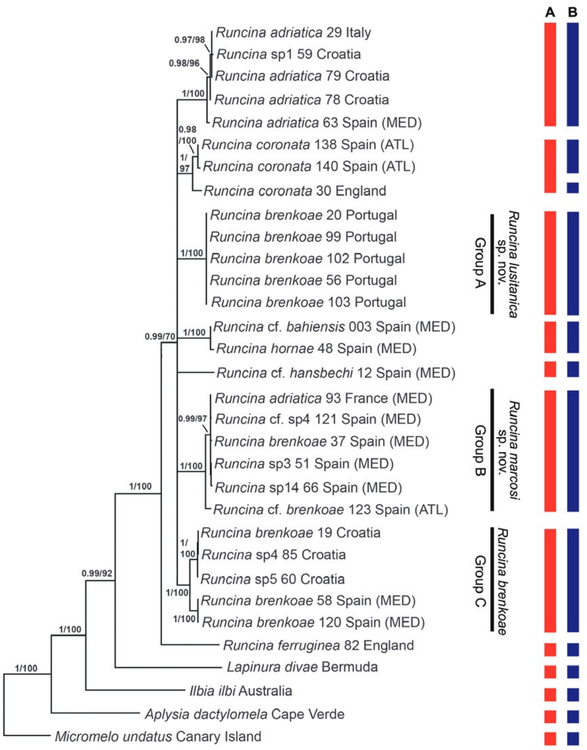

Fig. 1. – Phylogenetic hypothesis based on the combined dataset (H3+COI+16S) inferred by Bayesian analysis. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Numbers after the sequence name refer to individual specimen numbers. Abbreviations: ATL, Atlantic Ocean; MED, Mediterranean Sea. A, ABGD based on the COI data set; B, bPTP result based on the COI and 16S data sets.

Table 2. – Uncorrected *p*-distances based on COI sequences.

	Distance between groups (%)		Distance within groups (%)
	Group A	Group B	
Group A			0.0
Group B	11.7-12.0		0.0-4.0
Group C	9.6-11.6	10.3-1.5	0.0-4.6

0% within specimens of Group A, 4% within Group B, and 4.6% within Group C (Table 2). Between species in the genus *Runcina* the COI uncorrected *p*-distances ranged from 9.3% to 15.1%, while between the genera *Runcina* and *Lapinura* they ranged from 16.3% to 20.7%. No COI gene sequences from *Ilbia ilbi* were available for this analysis. The results obtained with the bPTP analysis were congruent with the ABGD output in suggesting the same three groups of *Runcina brenkoae* (Fig. 1B).

The molecular results support the occurrence of three species under the name *Runcina brenkoae*, and this hypothesis is backed by morphological differences across specimens from the three *R. brenkoae* clades (see Systematic description section). Therefore, we present below a redescription of *R. brenkoae* and we describe two new species.

SYSTEMATIC DESCRIPTION

Family RUNCINIDAE H. Adams and A. Adams, 1854
Genus *Runcina* Forbes in Forbes and Hanley, 1851

Runcina brenkoae Thompson, 1980
(Figs 2, 5A-C, 6A, D)

Runcina brenkoae Thompson 1980: 156, fig. 1C. Thompson and Brodie 1988: 340, fig. 1D. Schmekel and Capellato 2001: 144, Pl. I g; 145, Pl. II k; and 148, Pl. III a, b; Schmekel and Capellato 2002: 98, Pl. VI a-c. Ballesteros et al. 2016: 4, fig. 7A.

Type material. Holotype (NHMUK 197913W) Natural History Museum, London, UK (not studied because the material is only available as micro-slide preparations).

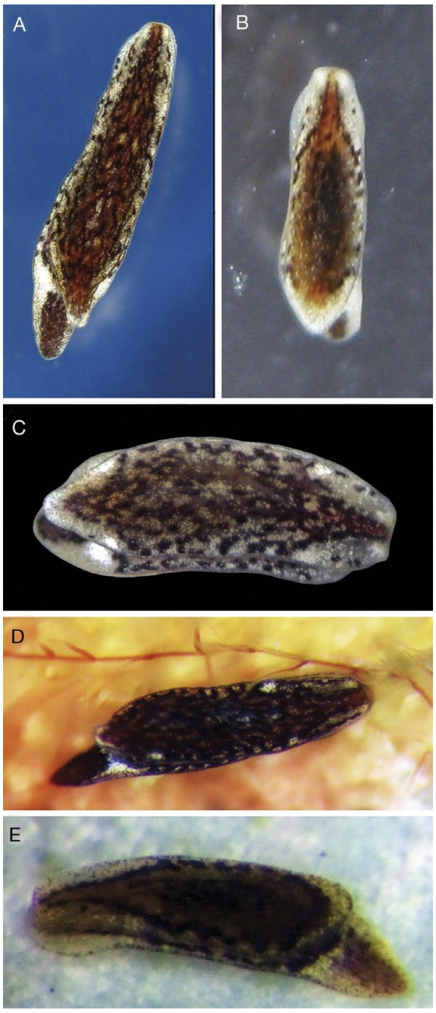

Fig. 2. – Living animals of *Runcina brenkoae*. A (MNCN 15.05/88086), B (MNCN 15.05/88089), C (MNCN 15.05/88088); specimens from Croatia (Adriatic Sea) (photos Alen Petani). D (MNCN 15.05/88087), E (MNCN 15.05/88090); specimens from Catalonia, Spain (Mediterranean) (photos Marina Poddubetskaia).

Type locality. Rovinj, Croatia.

Examined material. (MNCN 15.05/88086): Split, Croatia, 03 Aug 2014, 1.5 mm in length preserved, depth 1 m. Found washing *Posidonia* (dissected and sequenced). (MNCN 15.05/88087): Roses, Catalonia, Spain, coll. Marina Poddubetskaia, 08 Aug 2016, 1 mm in length preserved, depth 8 mm. Found on *Posidonia* (dissected, sequenced). (MNCN 15.05/88088): Nin, Croatia, coll. Alen Petani, 04 Apr 2017, 3.5 mm in length preserved, depth 0.5-1 m (dissected and sequenced). (MNCN 15.05/88090): Roses, Catalonia, Spain, coll. Marina Poddubetskaia, 19 Jul 2017, 1 mm in length preserved, depth 9 m (sequenced). (MNCN 15.05/88089): Nin, Croatia, coll. Alen Petani, 26 Dic 2017, 1.5 mm in length preserved, depth 0-1 m (sequenced).

External morphology (Fig. 2). Body moderately elongated and tapered. Notum smooth. Foot as wide as notum, showing a developed median pallial crest. Ground colour of body red-brown, sometimes translucent pale fawn bearing a pattern of anastomosing dark blotches on notum, margin and sole of foot. Eyes difficult to discern. Chalk-white spots all over body, more concentrated on margin of tail, both sides of head behind eyes and on metapodium in front of dark band. Some specimens with small red spots on margin of tail and surface of metapodium. The slugs have a longitudinal band of dark brown or wine-red colour on the surface of the metapodium. Two equal-sized translucent gills with white spots bearing pinnules on right posterior side of body. Anal pore situated beneath gills.

Internal anatomy (Figs 5A-C, 6A, D). Radular formulae 20 × 1.1.1 (MNCN 15.05/88086, MNCN 15.05/88088). Rachidian tooth boomerang-shaped with long, smooth lateral wings on each side. Central part of rachidian tooth bilobed; masticatory edge contains a pair of cockle-shaped rounded pads, each pad with 8-10 denticles. Median deep and broad depression is present between the pads; a small denticle may be present (Fig. 5A). Lateral teeth smooth, elongate and curved like a swan neck (Fig. 5B). Triangular jaws present. Four gizzard plates with 5-7 lamellae (Fig. 5C). Shell absent. Reproductive system monaulic. Female gland mass slightly divided into two lobes. Common genital duct connecting the female gland to the exterior on right posterior side of the body. Bursa copulatrix absent. Female gland placed on right posterior side of digestive gland (Fig. 6A). Male copulatory organ opens to the right of the mouth. Short and unarmed penial papilla projects into the atrium. Prostate gland long and cylindrical. Slender seminal vesicle with half size of prostate gland (Fig. 6D).

Runcina lusitanica n. sp.

(Figs 3, 5D-F, 6B, E)

http://zoobank.org/FAECCA78-B65B-47E6-8081-B2ABA0020F70

Examined material. Holotype (MNCN 15.05/200065): Near Faro, Algarve, Portugal, coll. Jorge Antonio Domínguez Godino, May 2015, 4 mm in length preserved (dissected and sequenced). Paratypes (MNCN 15.05/88091): Near Faro, Algarve, Portugal, coll. Jorge Antonio Domínguez Godino, May 2015, 5 mm in length preserved (dissected and sequenced). (MNCN 15.05/88092): Near Faro, Algarve, Portugal, coll. Jorge Antonio Domínguez Godino, May 2015, 5 mm in length preserved (dissected and sequenced). (MNCN 15.05/88093): Near Faro, Algarve, Portugal, coll. Jorge Antonio Domínguez Godino, May 2015, 4.5 mm in length preserved (dissected and sequenced). (MNCN 15.05/88094): Near Faro, Algarve, Portugal, coll. Jorge Antonio Domínguez Godino, May 2015, 4.5 mm in length preserved (dissected and sequenced).

Etymology. Lusitania was the name of a Roman province in the west of the Iberian Peninsula that occupied much of what now is Portugal.

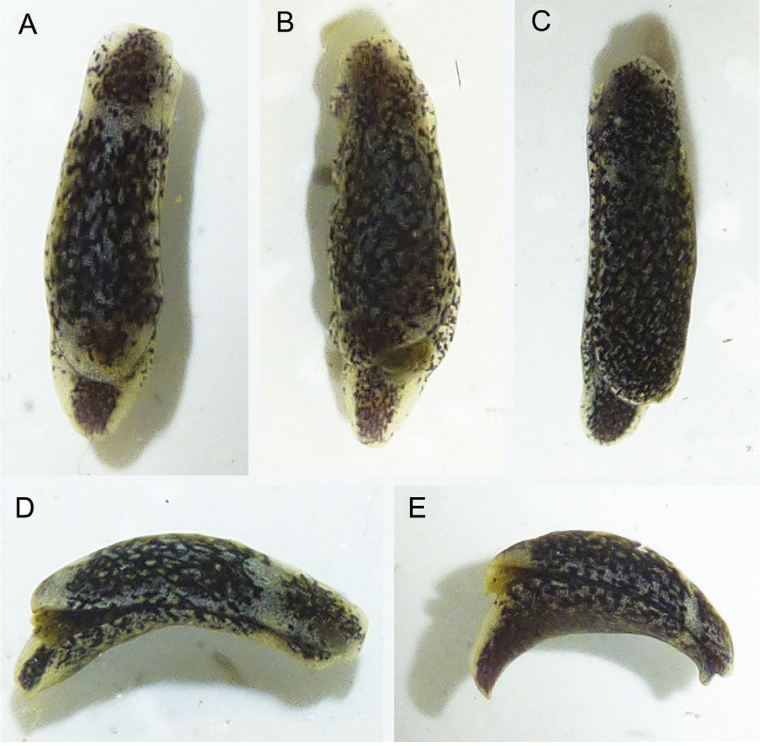

Fig. 3. – Living animals of *Runcina lusitanica* n. sp. (south coast of Portugal). A (MNCN 15.05/88092), D (MNCN 15.05/200065), E (MNCN 15.05/88094); specimens showing the absence of dark blotches on the posterior part of the head and notum. B (MNCN 15.05/88091), C (MNCN 15.05/88093); specimens with dark blotches covering the whole notum.

External morphology (Fig. 3). Body elongated and moderately broad. Notum smooth. Foot as wide as notum. Posterior part of notum rounded without pallial crest. Ground colour of body brown and translucent yellowish bearing a pattern of anastomosing dark blotches on notum and margin of foot. Some specimens have a large pale fawn patch on the posterior part of head and notum. Eyes not visible. White spots on some specimens. Longitudinal band, sometimes wide, of dark brown colour on surface of metapodium. Two large, yellowish gills with dark spots bearing irregular pinnules on right posterior side of body. Upper gill unipinnate and the most ventral bipinnate. Anal pore situated beneath gills.

Internal anatomy (Figs 5D-F, 6B, E). Radular formulae 25 × 1.1.1 (MNCN 15.05/88092) and 29 × 1.1.1 (MNCN 15.05/88093). Rachidian tooth boomerang shaped with one long and smooth lateral wing on each side. Central part of rachidian tooth bilobed; masticatory edge contains a pair of flat, comb-shaped pads, each one possessing 10-12 denticles. Median deep and broad depression is present between the pads; a small denticle present (Fig. 5D). Lateral teeth smooth, elongate and curved like a swan neck (Fig. 5E). Triangular jaws present. Four gizzard plates with 10-11 lamellae (Fig. 5F). Shell absent. Reproductive system monaulic. Female gland mass divided into two lobes, located on right side and behind the digestive gland. Bursa copulatrix absent. Common genital duct opening to exterior on right posterior side of body (Fig. 6B). Male copulatory organ comprises a relatively large atrium, which opens on right side next to mouth. Short, unarmed, conical penial papilla projects inside atrium. Long and cylindrical prostate gland. Elongated and convoluted seminal vesicle (Fig. 6E).

Runcina marcosi n. sp.
(Figs 4, 5G-I, 6C, F, G)

http://zoobank.org/1E0B605C-C403-41F4-881B-3439F2D9C41C

Examined material. Holotype (MNCN 15.05/200066): La Caleta (Cádiz), Andalusia, southwestern Spain, coll. Josep Romà, 17 May 2015, 2.5 mm in length preserved, depth 0.5 – 1 m. Found on samples

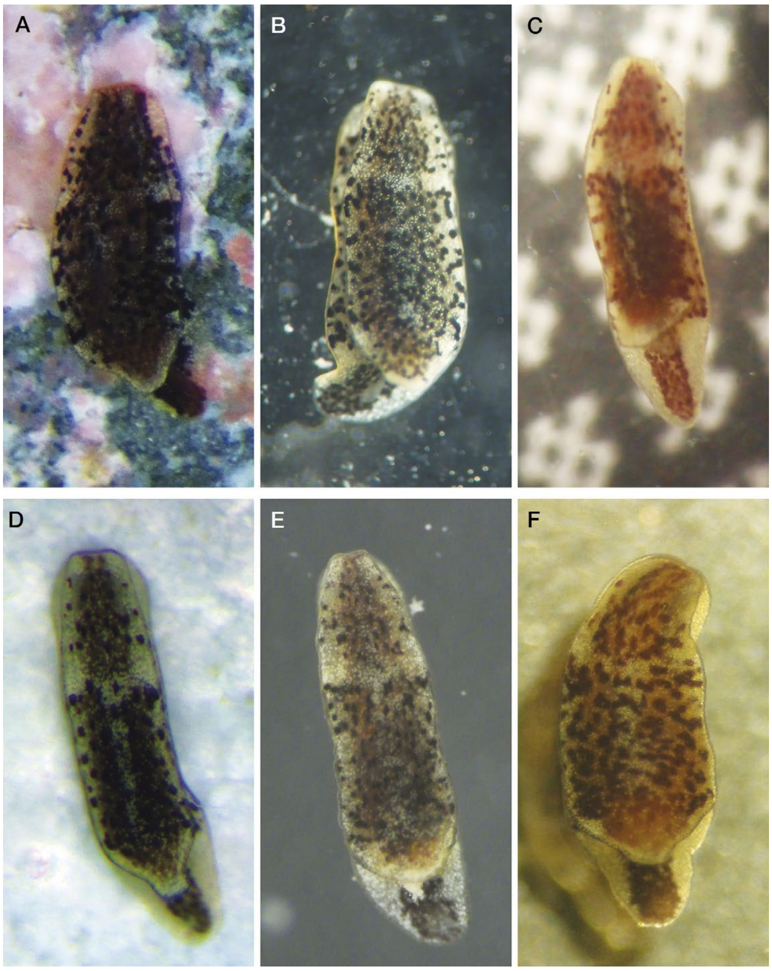

Fig. 4. – Living animals of *Runcina marcosi* n. sp. A (MNCN 15.05/88098), B (MNCN 15.05/88095), C (MNCN 15.05/88096), D (MNCN 15.05/88097); specimens from Catalonia, Spain (Mediterranean) (photos Marina Poddubetskaia, Ana Karla Araujo, Carles Galià). E (ZSM MOL 201442089); specimen from Banyuls-sur-Mer, France (Mediterranean) (photo Bastian Brenzinger). F (MNCN 15.05/200066); specimen from Cádiz, Spain (Atlantic) (photo Ana Karla Araujo).

of the brown algae *Halopteris scoparia* (dissected and sequenced). Paratypes (ZSM MOL 201442089): Banyuls-sur-Mer, France (Mediterranean), coll. Bastian Brenzinger and Timea Neusser, 02 Jul 2014, 1.3 mm in length preserved (sequenced). (MNCN 15.05/88095): Mataró, Catalonia, northeastern Spain, coll. Manuel Ballesteros, 22 Sep 2015, 1 mm in length preserved. Found on green algae (dissected and sequenced). (MNCN 15.05/88096): Roses, Catalonia, northeastern Spain, coll. Carles Galià, 29 May 2017, 1 mm in length preserved. Found on roots of seagrass *Posidonia oceanica* (dissected and sequenced). (MNCN 15.05/88098): Roses, Catalonia, northeastern Spain, coll. Marina Poddubetskaia, 30 Aug 2017, 1.5 mm in length preserved (dissected and sequenced). (MNCN 15.05/88097): Roses, Catalonia, northeastern Spain, coll. Marina Poddubetskaia, 19 Jul 2018, 2 mm in length preserved, depth 4 m (sequenced).

Etymology. This species is dedicated to Marcos Martínez Vazquez, husband of the first author, for all his help, enthusiasm and support during the course of this work.

External morphology (Fig. 4). Body moderately elongated. Notum smooth. Foot as wide as notum. Some specimens show developed median pallial crest. Ground colour of body red-brown or translucent pale fawn bearing a pattern of anastomosing dark or reddish blotches on notum, margin of foot and metapodium.

Eyes difficult to discern. White spots all over the body. Longitudinal band of dark brown or wine-red colour on surface of metapodium. Two translucent gills bearing regular pinnules on right posterior side of body. Upper gill unipinnate and the most ventral bipinnate. Anal pore situated beneath gills.

Internal anatomy (Figs 5G-I, 6C, F, G). Radular formulae 10 x 1.1.1 (MNCN 15.05/88097) and 13 × 1.1.1 (MNCN 15.05/88095). Rachidian tooth boomerang-shaped with long and smooth lateral wings on each side. Central part of rachidian tooth bilobed; masticatory edge contains a pair of flat, comb-shaped pads, each one with 10-11 denticles. Median deep and broad depression present between the pads; small denticle absent (Fig. 5G). Lateral teeth smooth, elongate and curved like a swan neck (Fig. 5H). Triangular jaws present. Four gizzard plates with 7-8 lamellae (Fig. 5I). Shell absent. Reproductive system monaulic. Female gland mass placed on right side and behind the digestive gland. Divided into two lobes, perhaps albumen and mucous glands. Long common genital duct connects the female gland to exterior on right posterior side of body. Bursa copulatrix absent (Fig. 6C). Elongated and cylindrical male copulatory organ. Atrium opens to right side of mouth. Short and unarmed penial papilla projects into the atrium. Cylindrical prostate gland. Slender seminal vesicle with half size of prostate gland (Fig. 6F, G).

DISCUSSION

Recent molecular studies on heterobranch sea slugs, mostly nudibranchs, have demonstrated the existence of many complexes of cryptic species (Austin et al. 2018, Layton et al. 2018, Korshunova et al. 2019, among many others). Up to now, most studies related to the order Runcinida have focused only on morphological aspects in order to identify and describe new species and genera (Cervera et al. 1991,

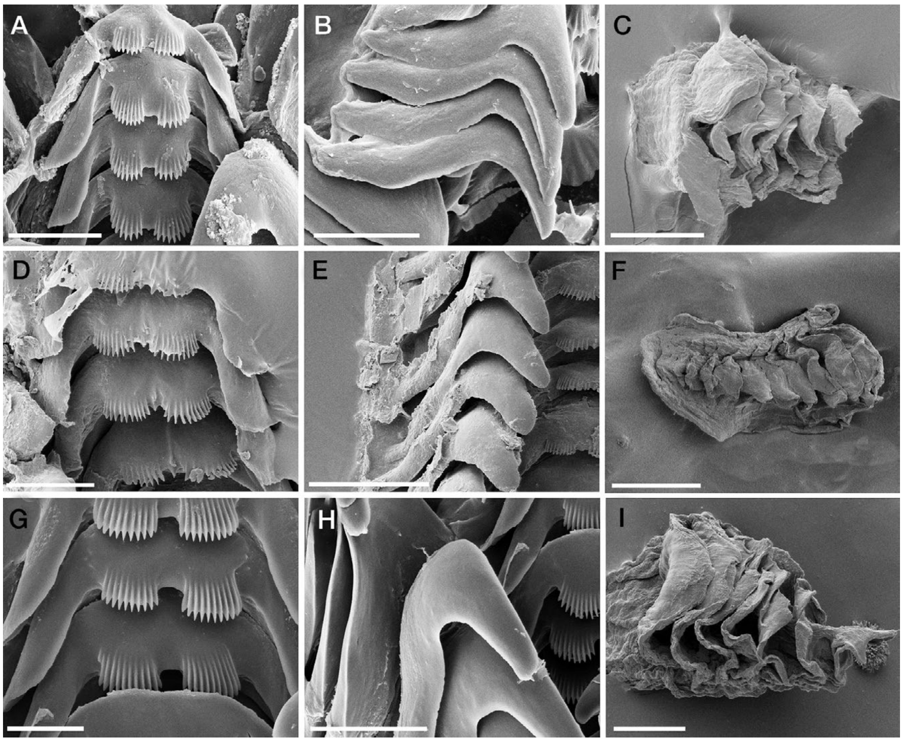

Fig. 5. – Scanning electron micrographs of jaw and radula of *Runcina* species. A, B, C, *R. brenkoae* (MNCN 15.05/88086, MNCN 15.05/88088): A, radular teeth (MNCN 15.05/88088); B, lateral teeth (MNCN 15.05/88086); C, Gizzard plate (MNCN 15.05/88088). D, E, F, *R. lusitanica* n. sp. (MNCN 15.05/88093): D, radular teeth; E, lateral teeth; F, Gizzard plate. G, H, I, *R. marcosi* n. sp. (MNCN 15.05/88095, MNCN 15.05/88097): G, radular teeth (MNCN 15.05/88095); H, lateral teeth (MNCN 15.05/88095); I, Gizzard plate (MNCN 15.05/88097). Scale bars: A, B, E, H=10 µm; C=50 µm; D, I=20 µm; F=100 µm; G=5 µm.

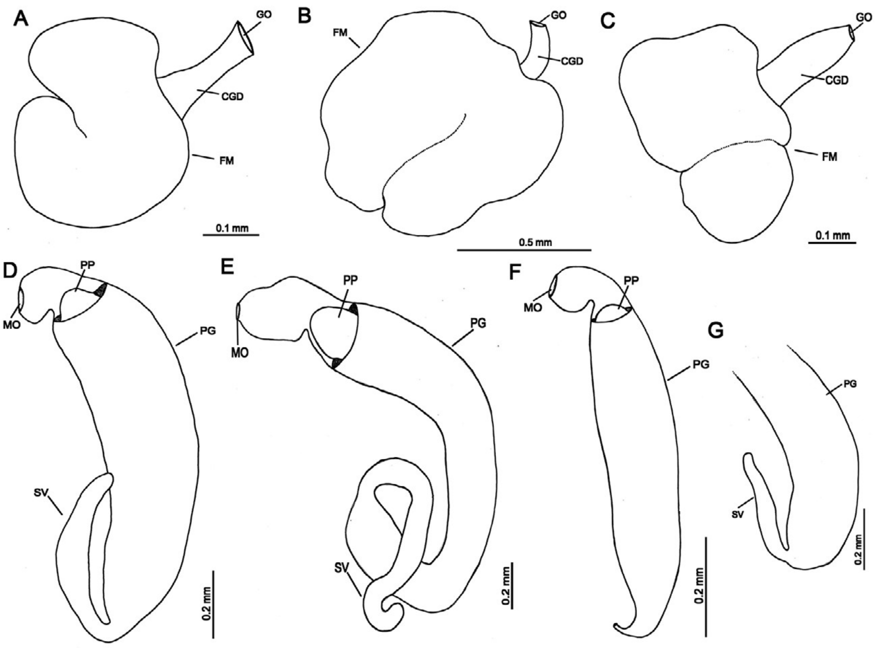

Fig. 6. – Reproductive system of *Runcina* species. Top row female part (A, B, C) and lower row male part (D, E, F, G). A, D, *R. brenkoae*, Croatia (MNCN 15.05/88088); B, E, *R. lusitanica* n. sp., Portugal (MNCN 15.05/88093); C, G, *R. marcosi* n. sp., Cádiz, Spain (MNCN 15.05/200066), male copulatory organ damaged without penial papilla, but showing seminal vesicle; F, *R. marcosi* n. sp., Catalonia, Spain (MNCN 15.05/88097), male copulatory organ damaged without seminal vesicle. Abbreviations: fm, female mass; cgd, common genital duct; go, gonopore; mo, male opening; pp, penial papilla; pg, prostate gland; sv, seminal vesicle.

Chernyshev 2006, Moro and Ortea 2015). Our contribution is the first to use molecular phylogenetics combined with morphology to test the status of taxonomically difficult European runcinids, with a focus on the *Runcina brenkoae* species complex. Our study recognized three distinct species within this complex, namely *R. brenkoae* Thompson, 1980 proper and two new species described here as *R. marcosi* n. sp. and *R. lusitanica* n. sp. (Table 3).

Externally, all species of this complex are similar in colour, but *R. marcosi* n. sp., despite its chromatic variability, has a characteristic concentration of white spots on the anterior part of the body forming a “necklace”. *R. brenkoae* is the only one among the three species of the complex with both gills unipinnate, whereas *R. lusitanica* n. sp. and *R. marcosi* n. sp. have one gill unipinnate and the other bipinnate. *R. lusitanica* n. sp. reaches comparatively larger sizes (up to 5 mm in length in preserved animals), but overlaps chromatically with *R. brenkoae*. *R. marcosi* n. sp. shows a considerable chromatic variation and, in fact, some individuals can be confused with *R. adriatica*, which has chalk-white spots on the pallial crest and behind the eyes forming a “necklace” (Thompson 1980, Thompson and Brodie 1988). However, *R. adriatica* has three gills (two bipinnate and one unipinnate) and a higher number of radular rows (21 × 1.1.1) (Thompson 1980).

Anatomically these species differ in subtle details of the radula and gizzard plates. The pads of the rachidian tooth are more oval in shape in *R. brenkoae*, as observed by Schmekel and Cappellato (2001, 2002), whereas in *R. marcosi* n. sp. and *R. lusitanica* n. sp. these pads are more flattened. In *R. lusitanica* n. sp. and *R. brenkoae*, a small denticle is present in the depression between the two pads, but it may be absent in some rows. The gizzard plates of *R. brenkoae* have 5–6 lamellae, while in *R. marcosi* n. sp. and *R. lusitanica* n. sp. they have 7-8 and 10-11 lamellae, respectively.

The male copulatory organ of the runcinids consists of a penial papilla projecting into an atrium, a prostate gland, and a seminal vesicle (Vayssière 1883, Kress 1977, Burn and Thompson 1998). The male copulatory organ does not differ much between *R. brenkoae* and *R. marcosi* n. sp. The prostate is more curved in *R. brenkoae* than in *R. marcosi* n. sp., and the seminal vesicle

Table 3. – Differences between *Runcina brenkoae*, *Runcina lusitanica* n. sp. and *Runcina marcosi* n. sp. Data after Thompson (1980), Thompson and Brodie (1988), Schmekel and Cappellato (2002) and present study.

	Runcina brenkoae	*Runcina lusitanica* n. sp.	*Runcina marcosi* n. sp.
Colour pattern	Body red-brown, sometimes translucent pale fawn. Anastomosing dark blotches on notum, margin and sole of foot. Chalk-white spots all over body, more concentrated on margin of tail, both sides of head behind eyes and on metapodium in front of the dark band. Longitudinal band of dark brown or wine-red colour on surface of metapodium.	Body brown and translucent yellowish. Anastomosing dark blotches on notum and margin of foot. Some specimens have a large pale fawn patch on posterior part of head and notum. Longitudinal dark brown band, sometimes wide, on surface of metapodium.	Body red-brown or translucent pale fawn. Anastomosing dark or reddish blotches on notum, margin of foot and metapodium. White spots all over body. Longitudinal band of dark brown or wine-red colour on the surface of metapodium.
Gills	Two equal-sized translucent gills with white spots bearing pinnules.	Two large yellowish gills with dark spots bearing irregular pinnules. Upper gill unipinnate and ventral bipinnate.	Two translucent gills. Upper unipinnate and ventral bipinnate.
Shell	Absent	Absent	Absent
Radular formulae	20 × 1.1.1	25-29 × 1.1.1	10-13 × 1.1.1
Radula teeth	Rachidian tooth bilobed. Two pads cockle-shaped with 8-10 denticles each. Lateral teeth smooth, elongate and curved like a swan's neck.	Rachidian tooth bilobed. Two flat pads with 10-12 denticles each. Lateral teeth smooth, elongate and curved like a swan's neck.	Rachidian tooth bilobed. Two flat, comb-shaped pads with 10-11 denticles each. Lateral teeth smooth, elongate and curved like a swan's neck.
Gizzard plate	Four plates with 5-6 crests	Four plates with 10-11 crests	Four plates with 7-8 crests

in *R. brenkoae* is more rounded on one of the sides. Thompson (1980) did not mention any aspect of the male organ of *R. brenkoae*, nor did Thompson and Brodie (1988), and Schmekel and Cappellato (2002) only reported that the copulatory organ of *R. brenkoae* was similar to that of *R. ferruginea*, which has the same basic anatomical structure as the species described here. In *R. lusitanica* n. sp. the penial papilla is larger than in *R. brenkoae* and *R. marcosi* and the posterior end of the cylindrical prostate narrows slightly into a very long and twisted seminal vesicle, which is not present in *R. brenkoae* and *R. marcosi* n. sp.

The female part of the reproductive system in runcinids consists of an albumen and mucous gland opening to the outside through a common genital duct (Vayssière 1883, Kress 1977, Burn and Thompson 1998). However, the presence of an ampulla and bursa copulatrix have been described for the species *Runcina macfarlandi* (Gosliner, 1991), *R. coronate* and *Ilbia ilbi*, among others (Vayssière 1883, Burn 1963, Gosliner 1991). All three species of the *R. brenkoae* complex have similar female glands and we were unable to recognize an ampulla and bursa copulatrix. In general, the female part of the reproductive system in runcinids is poorly studied and, for example, Thompson (1980), Thompson and Brodie (1988) and Schmekel and Cappellato (2002) never referred to it.

Our study suggests that the geographical distribution of *Runcina brenkoae* proper is restricted to the Adriatic Sea (Croatia) and to the western Mediterranean (Spain and France), where it overlaps with the species *R. marcosi* n. sp., at least in northeastern Spain (Mediterranean Sea). Schmekel and Cappellato (2001, 2002) referred to its presence in Banyuls-sur-Mer (French Mediterranean coast) but their specimens were initially fixed in formalin (Ronald Janssen, pers. comm., Senckenberg Research Institute and Natural History Museum) and could not be tested for DNA. Thus, under the present taxonomic scenario the identity of these samples remains doubtful. The species *R. lusitanica* n. sp. is so far only known from the southern coast of Portugal. The distribution of *R. marcosi* n. sp. is restricted to southwestern Spain (Atlantic) and the western Mediterranean (Spain and France).

The present study is the first to evaluate the taxonomy of European species of runcinids using DNA data and to expose the occurrence of cryptic diversity among previously well-established species. Runcinids are small animals on average less than 5 mm in length, mostly with dull colour patterns, which complicates their identification and taxonomy. Runcinids clearly lack and will benefit from a DNA barcoding and molecular phylogenetics approach that could characterize the species molecularly, establishing a framework for understanding the value of colour patterns and morphological characters and their systematics.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

The following supplementary material is available through the online version of this article and at the following link:
http://scimar.icm.csic.es/scimar/supplm/sm04907esm.pdf

Fig. S1. – Phylogenetic hypothesis based on BI of the H3 gene. Numbers on the left of the slash are posterior probabilities and those on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

Fig. S2. – Phylogenetic hypothesis based on BI of the COI gene. Numbers on the left of the slash are posterior probabilities and those on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

Fig. S3. – Phylogenetic hypothesis based on BI of the 16S gene. Numbers on the left of the slash are posterior probabilities and those on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

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To be or not to be? What molecules say about *Runcina brenkoae* Thompson, 1980 (Gastropoda: Heterobranchia: Runcinida)

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Supplementary material

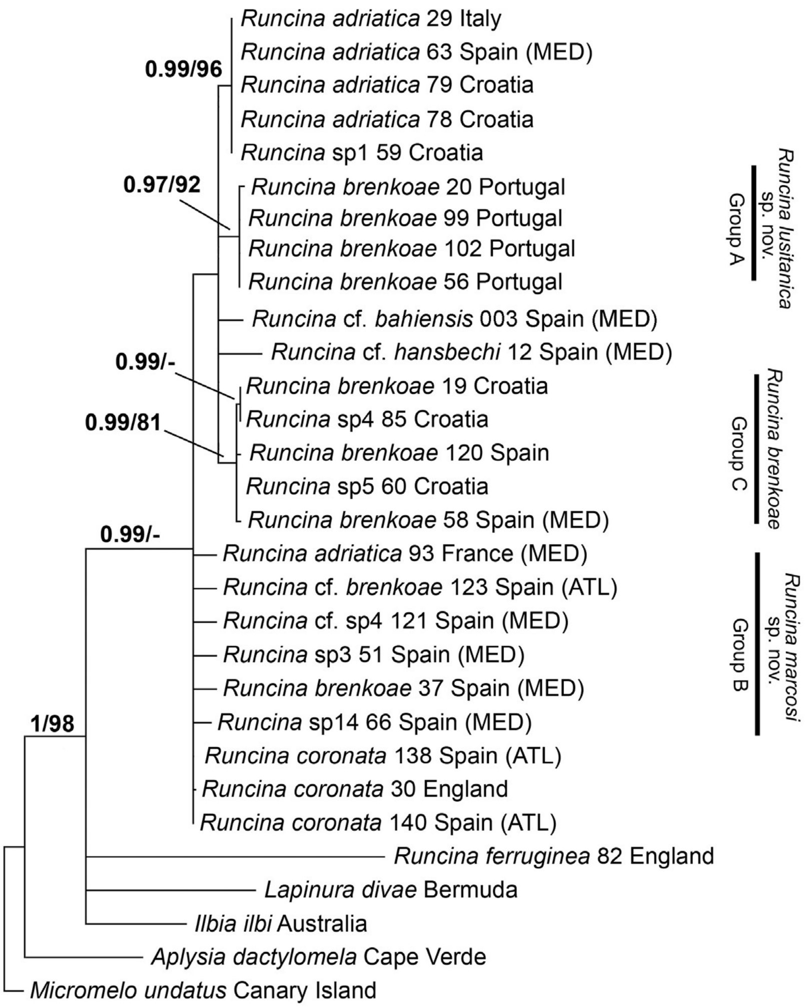

Fig. S1. – Phylogenetic hypothesis based on BI of the H3 gene. Numbers on the left of the slash are posterior probabilities and those on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

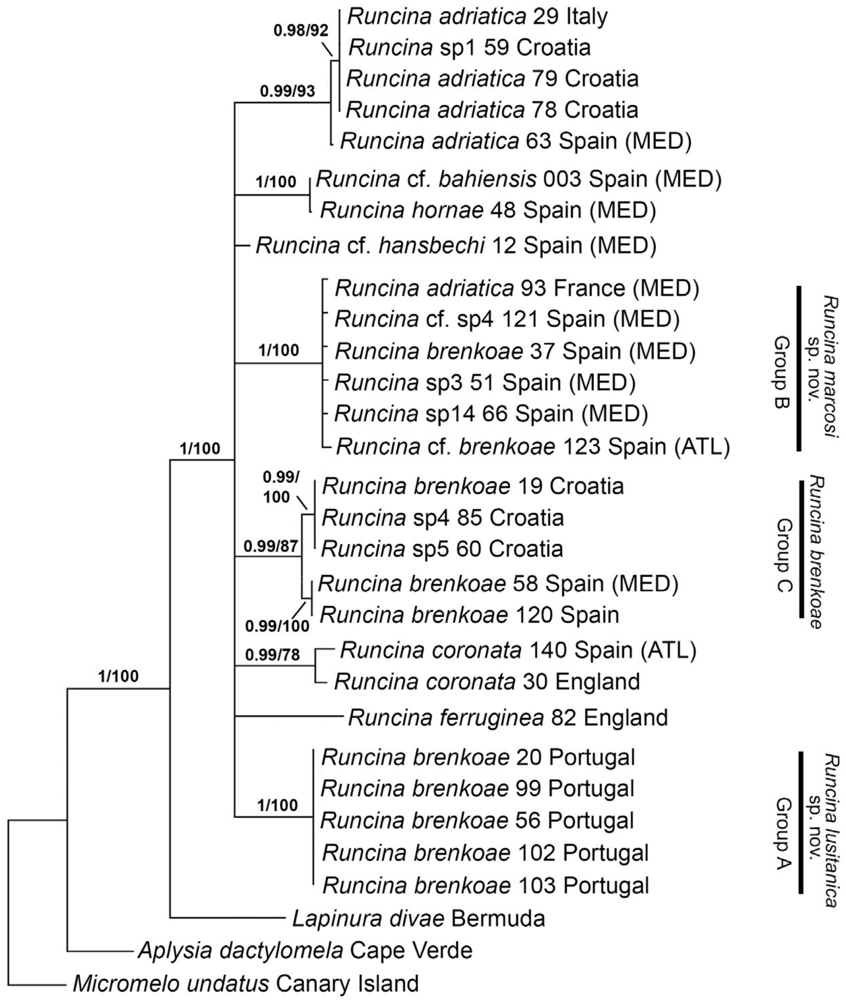

Fig. S2. – Phylogenetic hypothesis based on BI of the COI gene. Numbers on the left of the slash are posterior probabilities and those on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.

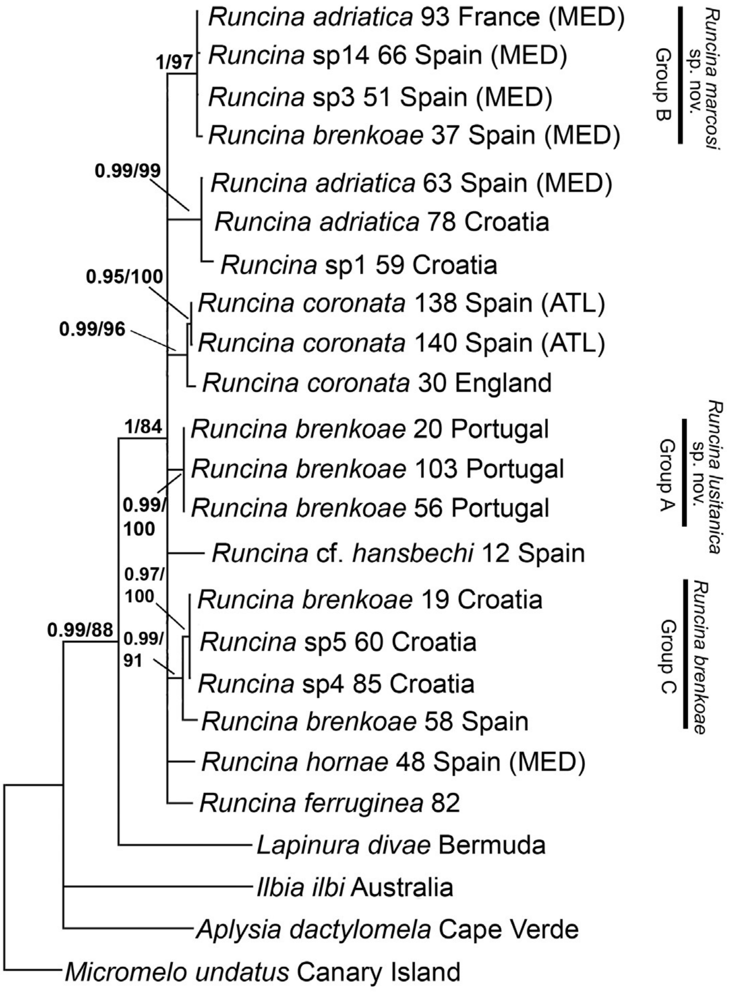

Fig. S3. – Phylogenetic hypothesis based on BI of the 16S gene. Numbers on the left of the slash are posterior probabilities and those on the right bootstrap values derived from maximum likelihood. Unsupported branches not labelled.