

To be or not to be? What molecules say about *Runcina brenkoe* 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 brenkoe*, 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. brenkoe* 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. brenkoe*, namely two new species here described (*R. marcosi* n. sp. and *R. lusitanica* n. sp.) and *R. brenkoe* proper.

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

¿Ser o no ser? Que dicen las moléculas sobre *Runcina brenkoe* Thompson, 1980 (Gastropoda: Heterobranchia: 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 brenkoe* 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. brenkoe* 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. brenkoe*, a saber, dos nuevas especies aquí descritas (*R. marcosi* n. sp. y *R. lusitanica* n. sp.) y *R. brenkoe* 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 Banyuls-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 con-

tributed 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 acteonid *Micromelo undatus* (Bruguière, 1792) and the aplousiid *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
<i>Ilbia ilbi</i> Burn, 1963		Hamers Haven, Australia	NMVF234189	MK322992*	-	MK323019*
<i>Lapinura divae</i> (Ev. Marcus and Er. Marcus, 1963)		Bermuda (GB)	ZMBN 82997	KJ022893	KF992195	KJ022825
<i>Runcina</i> sp. 1	<i>Runcina adriatica</i> T. Thompson, 1980	Iz Island, Croatia	MNCN 15.05/88099	MK323015*	MK322986*	MK323036*
<i>Runcina adriatica</i> T. Thompson, 1980		Ugljan Island, Croatia	MNCN 15.05/88100	MK322996*	MK322967*	MK323022*
<i>Runcina adriatica</i>		Zut Island, Croatia	MNCN 15.05/88101	MK322997*	MK322968*	-
<i>Runcina adriatica</i>		Otranto, Italy	MNCN 15.05/88102	MK322993*	MK322966*	-
<i>Runcina adriatica</i>		Cadaqués, Spain	MNCN 15.05/88103	MK322994*	MK322987*	MK323020*
<i>Runcina adriatica</i>	<i>Runcina marcosi</i> n. sp.	Banyuls-sur-Mer, France	ZSM MOL 201442089	MK322995*	MK322991*	MK323021*
<i>Runcina brenkoae</i> T. Thompson, 1980		Split, Croatia	MNCN 15.05/88086	MK322998*	MK322969*	MK323023*
<i>Runcina</i> sp.4	<i>Runcina brenkoae</i>	Nin, Croatia	MNCN 15.05/88089	MK323017*	MK322972*	MK323038*
<i>Runcina</i> sp.5	<i>Runcina brenkoae</i>	Nin, Croatia	MNCN 15.05/88088	MK323018*	MK322971*	MK323039*
<i>Runcina brenkoae</i>		Roses, Spain	MNCN 15.05/88087	MK323001*	MK322970*	MK323026*
<i>Runcina brenkoae</i>		Roses, Spain	MNCN 15.05/88090	MK323003*	MK322964*	-
<i>Runcina brenkoae</i>	<i>Runcina marcosi</i> n. sp.	Roses, Spain	MNCN 15.05/88098	MK323000*	MK322982*	MK323025*
<i>Runcina</i> sp.3	<i>Runcina marcosi</i> n. sp.	Mataró, Spain	MNCN 15.05/88095	MK323016*	MK322983*	MK323037*
<i>Runcina</i> cf. sp.4	<i>Runcina marcosi</i> n. sp.	Roses, Spain	MNCN 15.05/88097	MK323012*	MK322990*	-
<i>Runcina</i> sp.14	<i>Runcina marcosi</i> n. sp.	Roses, Spain	MNCN 15.05/88096	MK323013*	MK322984*	MK323034*
<i>Runcina</i> cf. <i>brenkoae</i>	<i>Runcina marcosi</i> n. sp.	Cádiz, Spain	MNCN 15.05/200066	MK323006*	MK322985*	-
<i>Runcina brenkoae</i>	<i>Runcina lusitanica</i> n. sp.	Faro, Portugal	MNCN 15.05/88091	MK322999*	MK322979*	MK323024*
<i>Runcina brenkoae</i>	<i>Runcina lusitanica</i> n. sp.	Faro, Portugal	MNCN 15.05/200065	MK323002*	MK322981*	-
<i>Runcina brenkoae</i>	<i>Runcina lusitanica</i> n. sp.	Faro, Portugal	MNCN 15.05/88093	MK323004*	MK322965*	-
<i>Runcina brenkoae</i>	<i>Runcina lusitanica</i> n. sp.	Faro, Portugal	MNCN 15.05/88092	MK323014*	MK322980*	MK323035*
<i>Runcina brenkoae</i>	<i>Runcina lusitanica</i> n. sp.	Faro, Portugal	MNCN 15.05/88094	-	MK322989*	MK323027*
<i>Runcina</i> cf. <i>bahiensis</i> Cervera, Garcia-Gomez and Garcia, 1991		Cadaqués, Spain	MNCN 15.05/88104	MK323005*	MK322988*	-
<i>Runcina coronata</i> (Quatrefages, 1844)		Swanage, England	MNCN 15.05/88105	MK323010*	MK322976*	MK323031*
<i>Runcina coronata</i>		Cádiz, Spain	MNCN 15.05/88106	MN057641*	-	MN057638*
<i>Runcina coronata</i>		Cádiz, Spain	MNCN 15.05/88107	MN057642*	MN057640*	MN057639*
<i>Runcina</i> cf. <i>hansbechi</i> Schmeckel and Cappellato, 2001		Mataró, Spain	MNCN 15.05/88108	MK32300*	MK322973*	MK323028*
<i>Runcina ferruginea</i> Kress, 1977		Newlyn, Cornwall, England	MNCN 15.05/88109	MK323011*	MK322977*	MK323032*
<i>Runcina hornae</i> Schmeckel and Cappellato, 2002		Palamós, Spain	MNCN 15.05/88110	-	MK322978*	MK323033*
<i>Aplysia dactylomela</i> Rang, 1828		Cape Verde (GB)	NHMUK 20030795/20030796	KJ022921	KF992168	KJ022798
<i>Micromelo undatus</i> (Bruguère, 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 94°C, 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 (Kato 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) ≥ 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 brenkoe* 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. brenkoe* 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. brenkoe* 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. brenkoe* 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

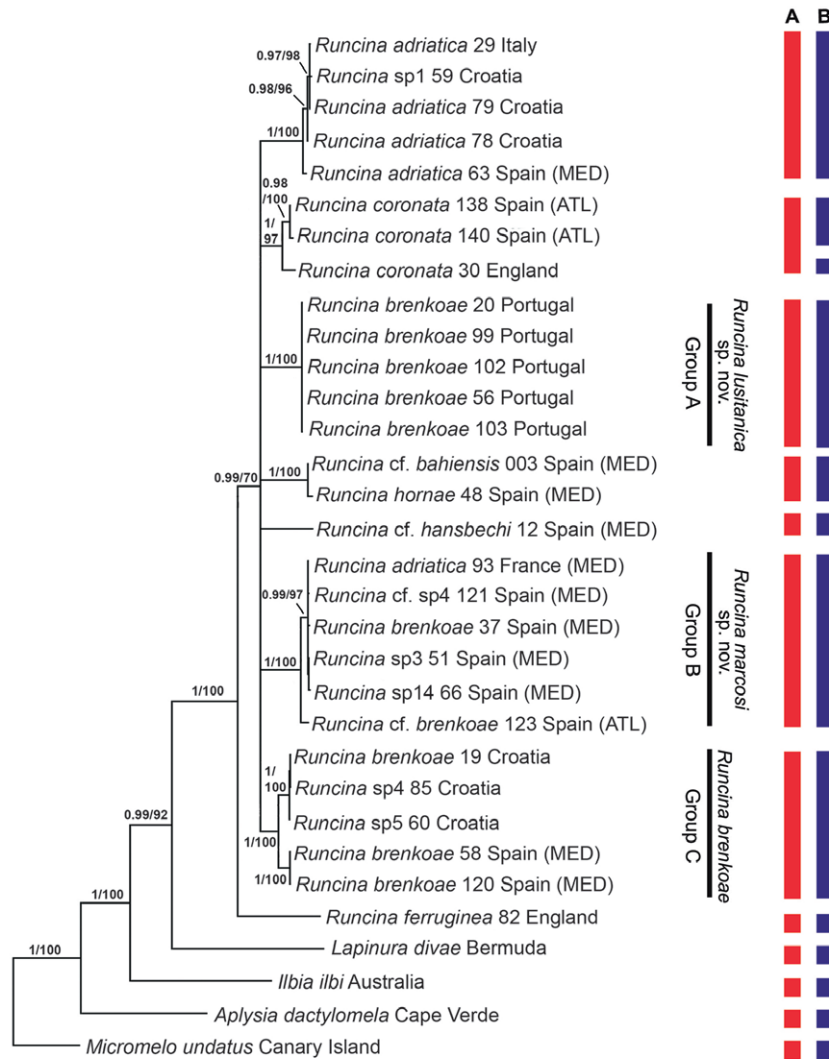


Fig. 1. – Phylogenetic hypothesis based on the combined dataset (H3+COI+16S) inferred by Bayesian analysis. Numbers on the left of the slash are posterior probabilities and on the right bootstrap values derived from maximum likelihood. Numbers after the sequence name refer to individual specimen numbers. 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).

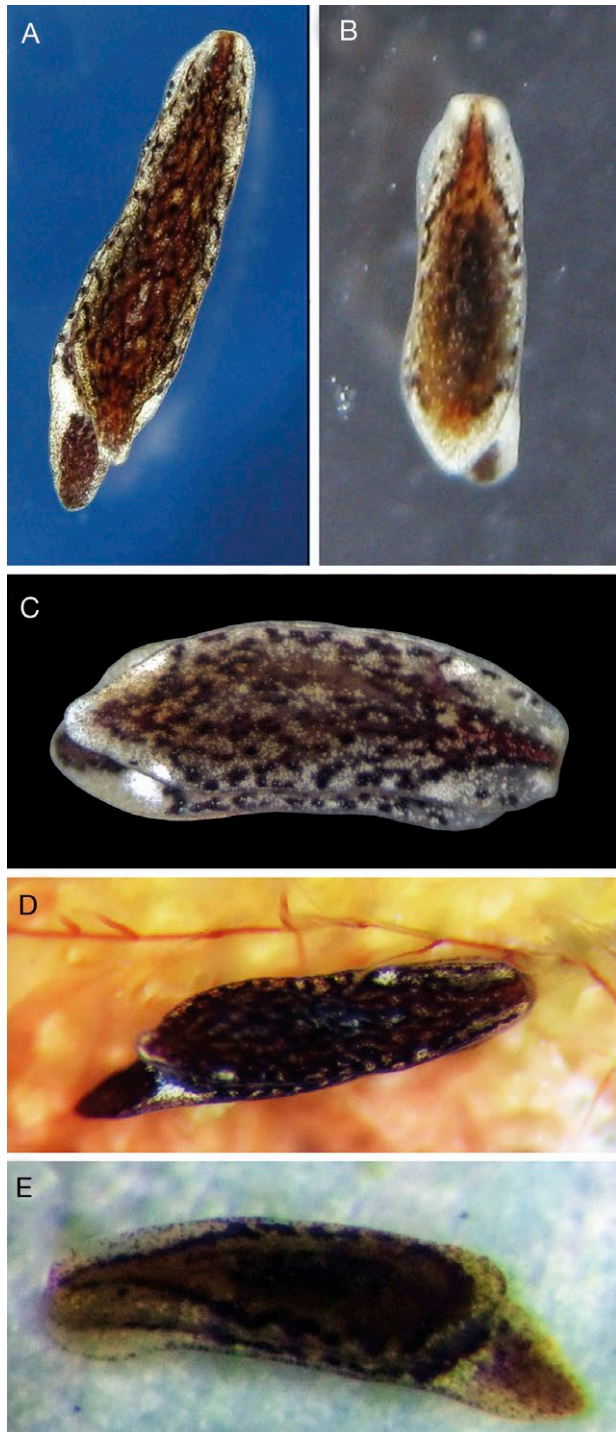


Fig. 2. – Living animals of *Runcina brenkoae*. A (MNCN 15.05/88086), B (MNCN 15.05/88089), C (MNCN 15.05/88088); specimens from Croatia (Adriatic Sea) (photos Alen Petani). D (MNCN 15.05/88087), E (MNCN 15.05/88090); specimens from Catalonia, Spain (Mediterranean) (photos Marina Poddubetskaia).

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 $20 \times 1.1.1$ (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.

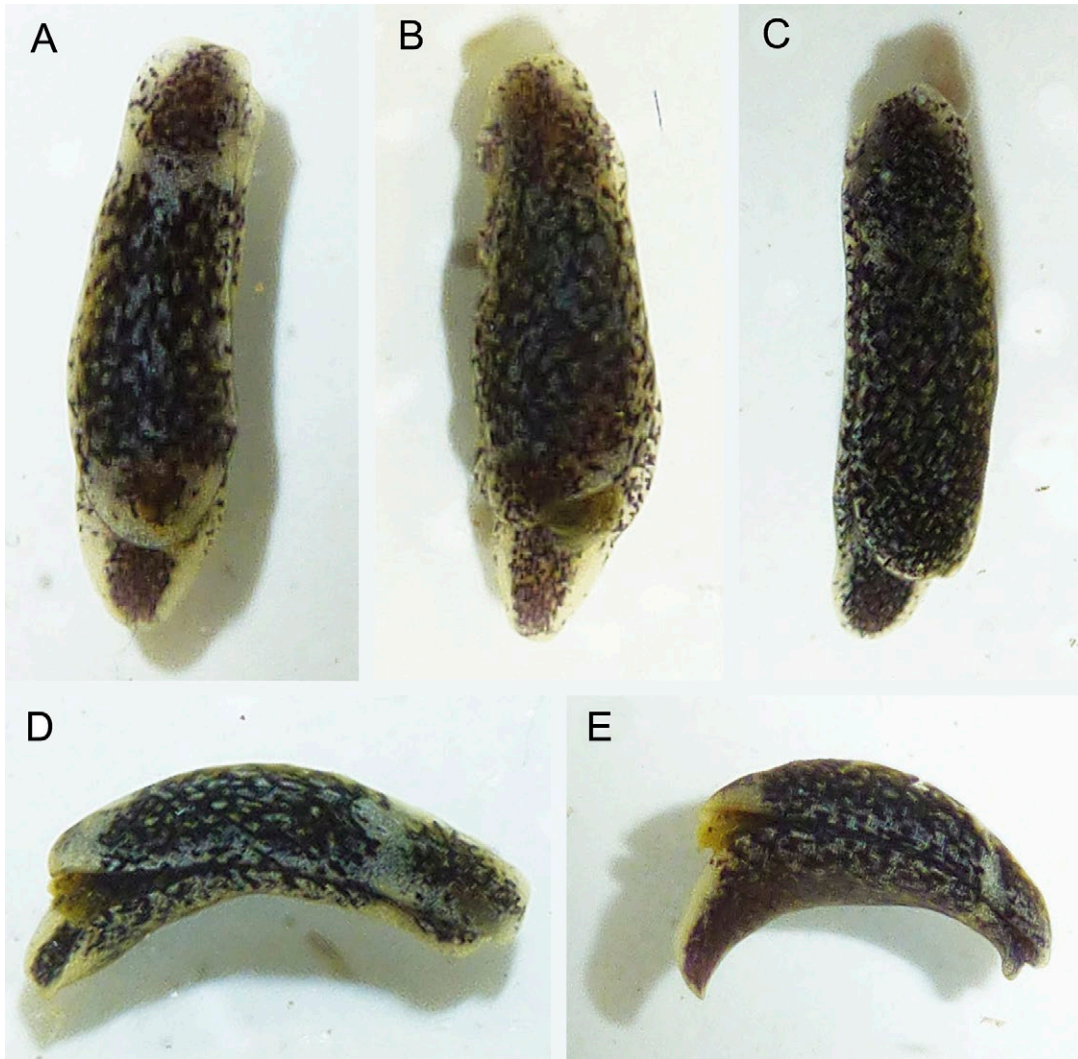


Fig. 3. – Living animals of *Runcina lusitanica* n. sp. (south coast of Portugal). A (MNCN 15.05/88092), D (MNCN 15.05/200065), E (MNCN 15.05/88094); specimens showing the absence of dark blotches on the posterior part of the head and notum. B (MNCN 15.05/88091), C (MNCN 15.05/88093); specimens with 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 $25 \times 1.1.1$ (MNCN 15.05/88092) and $29 \times 1.1.1$ (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

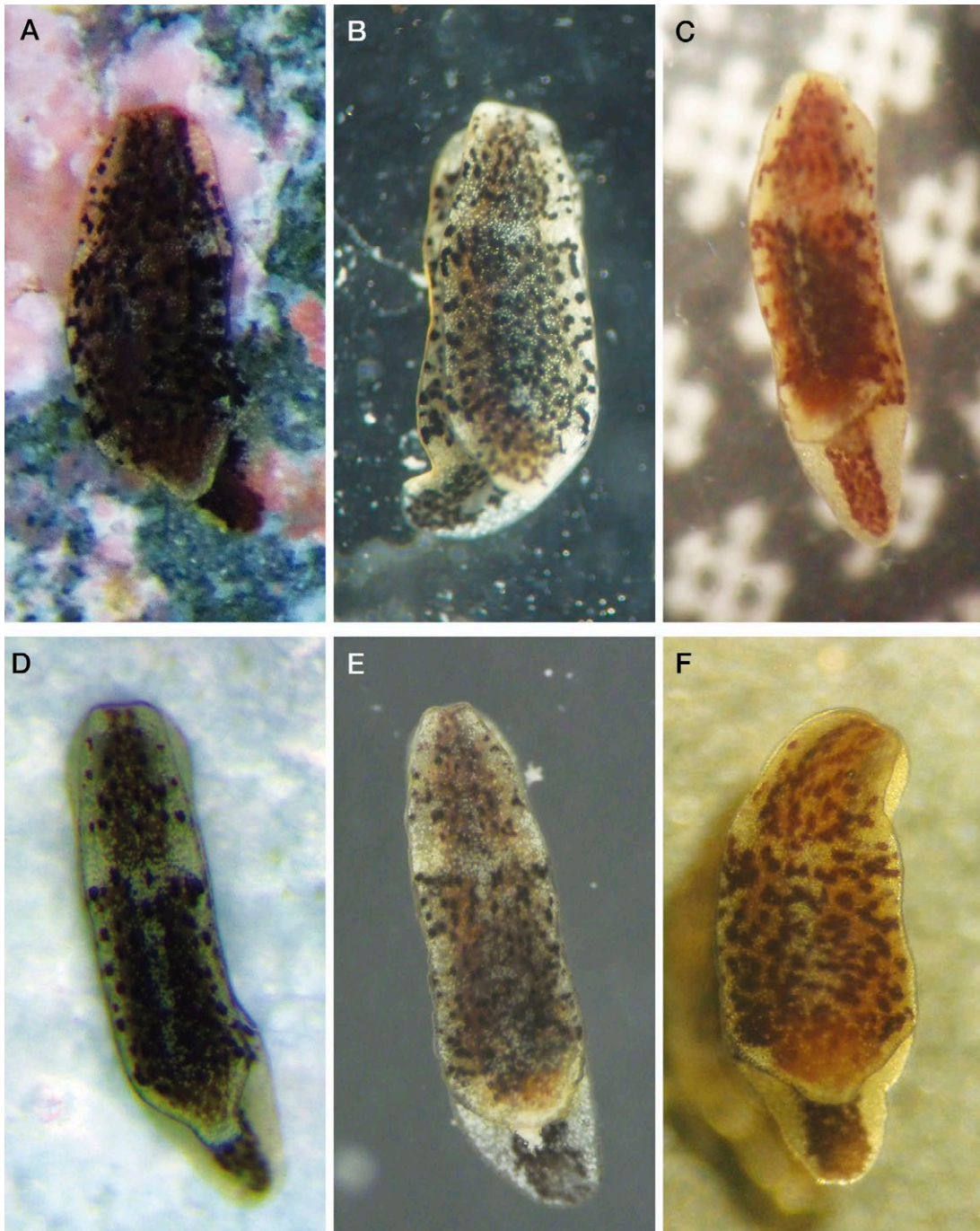


Fig. 4. – Living animals of *Runcina marcosi* n. sp. A (MNCN 15.05/88098), B (MNCN 15.05/88095), C (MNCN 15.05/88096), D (MNCN 15.05/88097); specimens from Catalonia, Spain (Mediterranean) (photos Marina Poddubetskaia, Ana Karla Araujo, Carles Galià). E (ZSM MOL 201442089); specimen from Banyuls-sur-Mer,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

tive 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,

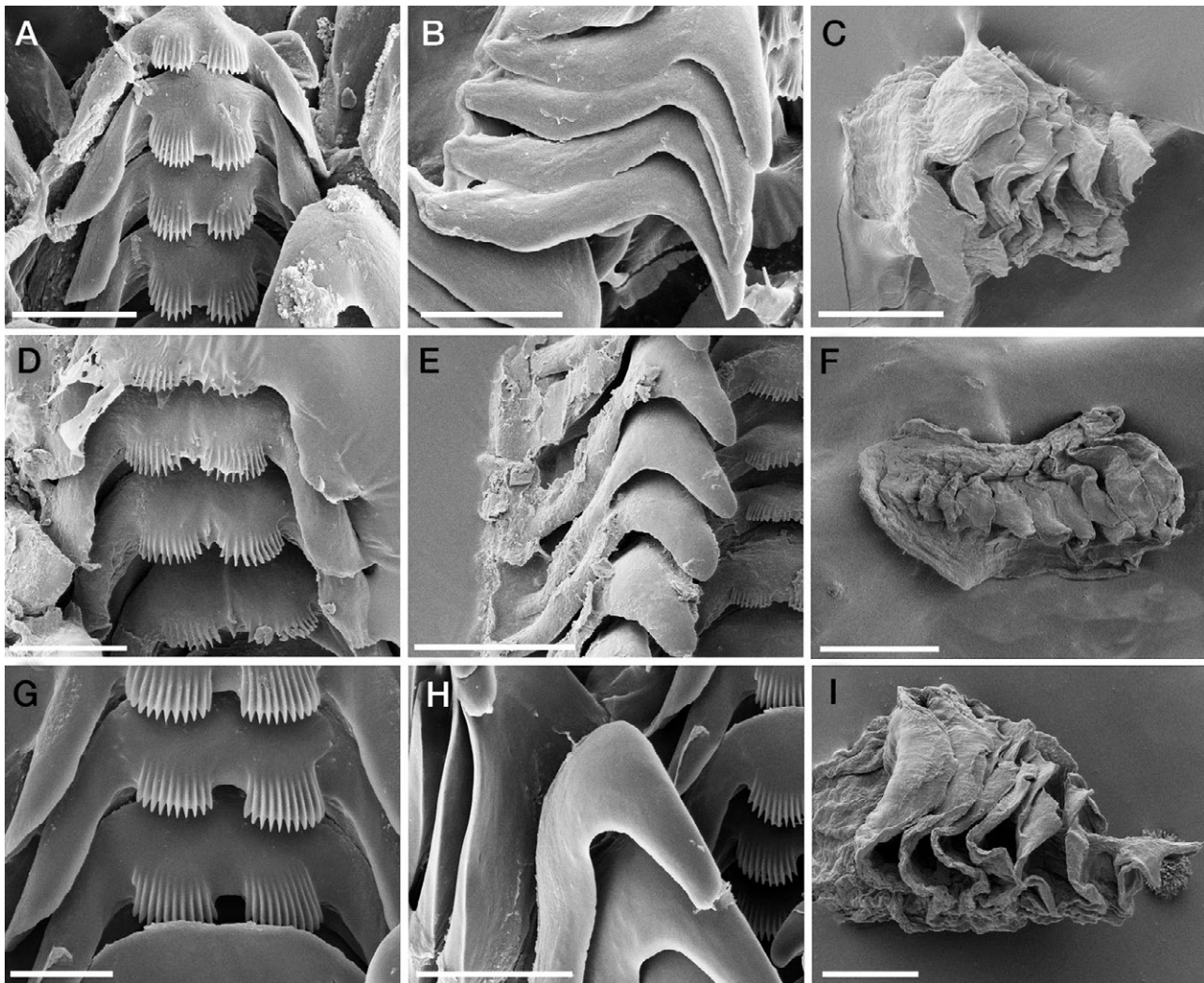


Fig. 5. – Scanning electron micrographs of jaw and radula of *Runcina* species. A, B, C, *R. brenkoae* (MNCN 15.05/88086, MNCN 15.05/88088): A, radular teeth (MNCN 15.05/88088); B, lateral teeth (MNCN 15.05/88086); C, Gizzard plate (MNCN 15.05/88088). D, E, F, *R. lusitanica* n. sp. (MNCN 15.05/88093): 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.

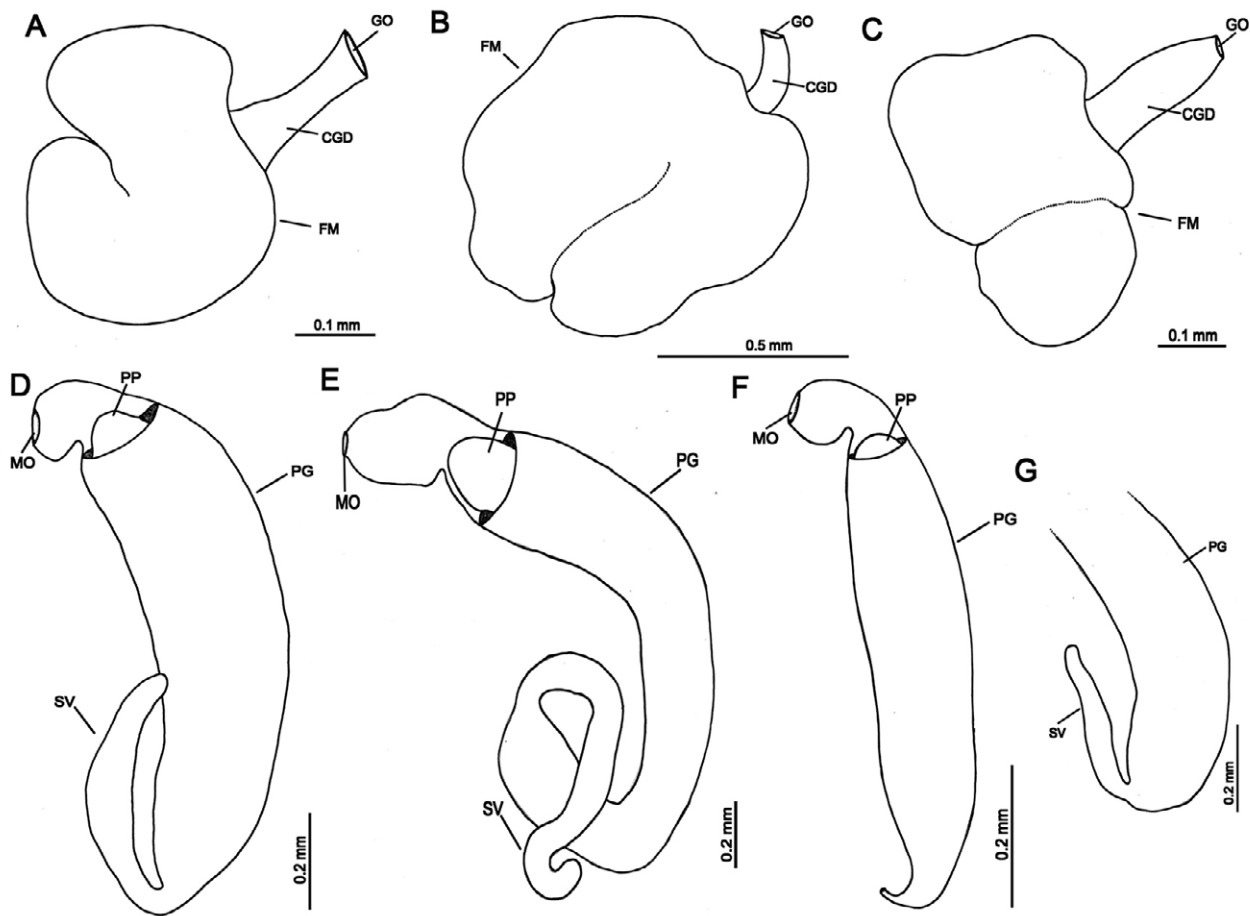


Fig. 6. – Reproductive system of *Runcina* species. Top row female part (A, B, C) and lower row male part (D, E, F, G). A, D, *R. brenkoae*, Croatia (MNCN 15.05/88088); B, E, *R. lusitanica* n. sp., Portugal (MNCN 15.05/88093); C, G, *R. marcosi* n. sp., Cádiz, Spain (MNCN 15.05/200066), male copulatory 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 ($21 \times 1.1.1$) (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.

	<i>Runcina brenkoae</i>	<i>Runcina lusitanica</i> n. sp.	<i>Runcina marcosi</i> 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 (Vayssi re 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 (Vayssi re 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 iden-

tity 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.

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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.