Acta Adriatica 64 (2023)
https://doi.org/10.32582/aa.64.2.1
ORIGINAL ARTICLE

www.izor.hr

Different but the same: DNA identification reveals a striking colour variability in a Mediterranean eolid sea slug specimen (Mollusca: Nudibranchia)

Giulia Furfaro[1,2*], Fabio Vitale[3,4], Alice Tagliabue[1], Cataldo Licchelli[4,5] and Paolo Mariottini[6]

[1] *Department of Science and Biological and Environmental Technologies - DiSTeBA, University of Salento, Lecce, Italy*
[2] *National Biodiversity Future Center (NBFC), Palermo, Italy*
[3] *Museum of Natural History of Salento, Calimera-Lecce, Italy*
[4] *Associazione Salento Sommerso, Lecce, Italy*
[5] *Cooperativa Hydra, Lecce, Italy*
[6] *Department of Science, University of 'Roma Tre', Rome, Italy*

Abstract: A peculiar eolid nudibranch showing an unknown chromatic array was found in a rocky bottom of Santa Maria al Bagno, in the Salento peninsula, Ionian Sea (Central Mediterranean Sea). This specimen, initially identified as *Piseinotecus* sp., was observed *in situ* and photographed while feeding and laying eggs close to individuals belonging to the Mediterranean *Piseinotecus soussi*. To assess the identity of this unexpected *Piseinotecus* 'white morph', a DNA identification approach was carried out using mitochondrial cytochrome c oxidase subunit I (COI), as it is the molecular marker mostly used to distinguish nudibranchs species. The molecular analysis unambiguously identified this specimen as *Piseinotecus soussi* and helped to shed lights on the striking intraspecific colour variability characterizing this rare species.
***Keywords*:** Piseinotecidae, *Piseinotecus soussi*, Intraspecific variability, Ionian Sea, Integrative taxonomy

Sažetak: RAZLIČIT, ALI ISTI: UZ POMOĆ DNA IDENTIFIKACIJE OTKRIVENA IZNENAĐUJUĆA OBOJENOST PRIMJERAKA SREDOZEMNOG MORSKOG PUŽA STRAŽNJOŠKRŽNJAKA (MOLLUSCA: NUDIBRANCHIA)**.** Neobični primjerak eolidnog morskog puža stražnjoškržnjaka s dosad nezabilježenim obojenjem pronađen je na stjenovitom dnu lokaliteta Santa Maria al Bagno na poluotoku Salentu u Jonskom moru (središnje Sredozemno more). Ovaj primjerak, prvobitno identificiran kao *Piseinotecus* sp., promatran je i fotografiran dok se hranio i polagao jaja u blizini jedinki koje pripadaju sredozemnoj vrsti *Piseinotecus soussi*. Kako bi se otkrio identitet ovog neobičnog „bijelog oblika" *Piseinotecus* sp. primjerka, provedena je identifikacija DNA pomoću mitohondrijske podjedinice citokrom c oksidaze I (COI), budući da je to molekularni marker koji se uglavnom koristi za razlikovanje vrsta stražnjoškržnjaka. Molekularna analiza nedvojbeno je identificirala ovaj primjerak kao *Piseinotecus soussi* i pomogla da se rasvijetli upečatljiva intraspecifična varijabilnost obojenja koja karakterizira ovu rijetku vrstu.
Ključne riječi: Piseinotecidae, *Piseinotecus soussi*, varijabilnost unutar vrste, Jonsko more, integrativna taksonomija

INTRODUCTION

Nudibranchia is a group of Gastropoda molluscs known for their brilliant and diversified colours. The latter are linked to characteristic defensive strategies that nudibranchs have evolved together with the loss of the shell in the adult stage. In fact, these sea slugs lose the protective shell after metamorphosis entrusting their protection to different systems like the ability to accumulate toxic or repellent bioactive compounds, the adoption of peculiar behaviours and being cryptic with the substrate they live on. Therefore, the colour in these organisms is particularly important and has always been considered a useful diagnostic character for recognizing different species. Body colour in nudibranchs has a strong link with species evolutionary history and can influence species fitness, although it was found to be misleading in some cases (Furfaro and Mariottini, 2016; Furfaro *et al*., 2021). The genus *Piseinotecus* Er. Marcus, 1955 has an Atlantic and Mediterranean distribution with only one species, *P. gonja* Edmunds, 1970 described from the western Indian Ocean (Tanzania). This genus is represented by the Brazilian *P. divae* Er. Marcus, 1955 (as the type species) and nowadays includes six accepted species*:* *P. ernestina* Ortea & Moro, 2020 from Cape Verde, the previously mentioned *P. gonja*, *P. minipapilla* Edmunds, 2015 from Ghana, *P. soussi* Tamsouri, Carmona, Moukrim and Cervera, 2014 and *P. sphaeripherus* (Schmekel, 1965), which has also been reported from the Mediterranean Sea. Unlike the latter, which has been recorded only a few times from

Corresponding author: giulia.furfaro@unisalento.it
Received: 17 March 2023, accepted: 29 May 2023
ISSN: 0001-5113, eISSN: 1846-0453

the Mediterranean Sea (Zenetos *et al*., 2016; Salvador *et al*., 2022) without collecting specimens or depositing molecular data, *P. soussi* was morphologically and molecularly investigated and its geographical distribution (from the eastern Atlantic Ocean to the Mediterranean Sea) confirmed (Tamsouri *et al*., 2014; Furfaro and Mariottini, 2019; Furfaro *et al*. 2020). Interestingly, this small aeolid is phenotypically very similar to both *Edmundsella albomaculata* (Pola, Carmona, Calado & Cervera, 2014), a flabellinid known only from São Vicente Island (Cape Verde), and *E. pedata* (Montagu, 1816), a very common Mediterranean flabellinid. Records of *P. soussi* in the Mediterranean basin are relatively few, most probably due to its small size, elusive behaviour (Furfaro and Mariottini 2019; Salvador *et al*., 2022), and possible misidentification with *E. pedata*, as already mentioned by Furfaro and Mariottini (2019).

The area of Santa Maria al Bagno, (Lecce, Italy), located in the Ionian Sea, is one of the four Mediterranean sampling localities where *P. soussi* occurrs (Tamsouri *et al*., 2014; Furfaro *et al*., 2020). This geographical spot encompasses a coastline of about 10 km, strongly influenced by the presence of karst cavities that have favoured the development of a very rich infralittoral biocenosis (Belmonte *et al*., 2010; Onorato and Belmonte, 2017). In this coastal stretch, wide cavities provide sciaphilic habitats that represent the ideal environment to favour the spreading of this taxon. *Piseinotecus soussi* has been annually observed during its breeding period, which in this Ionian area, due to its climatic conditions, takes place between the end of the winter and the beginning of spring (Furfaro *et al*., 2020). This aeolid is not very vagile and in Santa Caterina area it is common to observe it during mating or egg laying (Furfaro *et al*., 2020). During last year's underwater observations, the authors have recorded the presence of a peculiar specimen, showing a strikingly different body colour pattern, among *P. soussi* individuals. This individual opened questions about the possible occurrence of unknown diversity or the presence of a high chromatic variability within *P. soussi*. Therefore, we aimed to i) investigate the genetic identity of the 'white morph' specimen using DNA identification analysis, ii) unravel the different chromatic patterns that can occur during the development of *P. soussi* and iii) present an iconography of a striking colour phenotype.

MATERIALS AND METHODS

In situ observations and sampling were carried out by scuba diving in Santa Maria al Bagno, in the Salento peninsula (Lecce, Apulia, Italy; 40.1316282, 17.9963146; Fig. 1), at 5 m depth during the spring of 2019. The nudibranch was photographed underwater on 27th of April 2019, then collected and observed in the laboratory, preserved in 95% ethanol, and finally deposited with voucher RM3_2262 in the collection of the Department of Science of the Roma Tre University. Photos of the specimens and of the diagnostic characters (like the shape of the rhinophores, distribution and shape of the cerata, body colour pattern) were taken in daytime dive using specific equipment dedicated to underwater macro photography (Nikon D7000 with 60 mm micro Nikkor lens, with Isotta case and two Inon Z240 flashes).

Total genomic DNA was extracted from one individual by selecting a small piece of foot tissue and by using the 'salting out' procedure (Aljanabi and Martinez, 1997). The primer pair LCO1490 and HCO2198 (Folmer *et al*., 1994) was used for the amplification of the mitochondrial cytochrome oxidase subunit I (COI) gene, with following cycling parameters: 5 min of initial DNA denaturation at 94 °C; 35 cycles of 94 °C/30 s (DNA denaturation), 48 °C/60 s (annealing), 72 °C/60 s (elongation); and 7 min of final extension at 72 °C (Furfaro *et al*., 2016). The final volume of the PCR reaction was 20 µl. The amplified product was sequenced at the European Division of Macrogen Inc. (Amsterdam, The Netherlands). The COI sequence was edited with Staden Package 2.0.0b9 (Staden *et al*., 2000). BLASTN (Altschul *et al*., 1990) search was conducted in the GenBank database to confirm the identity of the sequenced

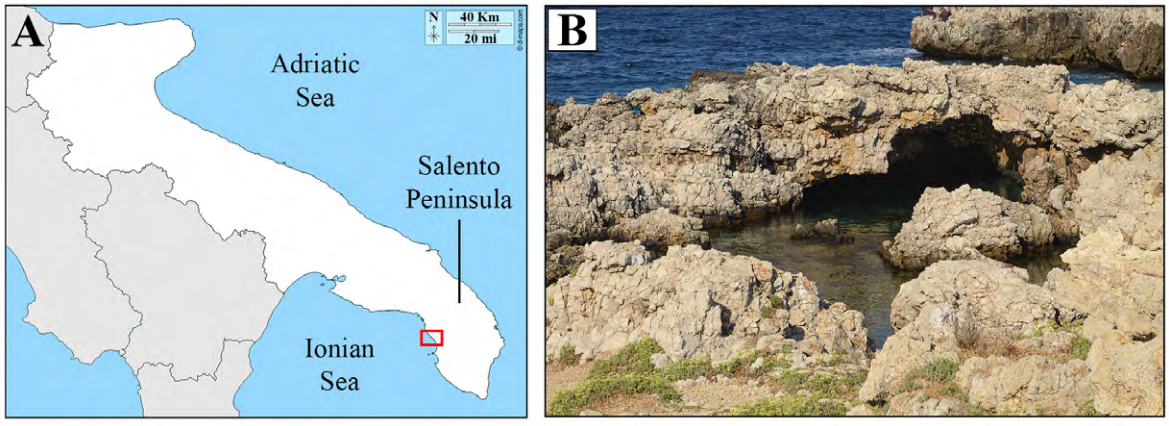

Fig. 1. Map of the collection locality in Apulian Salento peninsula, Ionian Sea (**A**) and external photograph of the diving site in Santa Maria al Bagno, in the Salento peninsula, Lecce, Italy (**B**).

Table 1. Collection locality, voucher, COI accession number from GenBank and reference of *Piseinotecus soussi* specimens here analysed.

Piseinotecus soussi	Locality	Voucher	COI Accession Number	References
'White morph'	Santa Maria, Apulia (Ionian Sea)	RM3_2262	OQ921890	Present study
'Typical morph'	Santa Maria, Apulia (Ionian Sea)	RM3_862	LR535941	Furfaro and Mariottini 2019
	Santa Maria, Apulia (Ionian Sea)	RM3_863	LR535942	Furfaro and Mariottini 2019
	Framura, Liguria (Tyrrhenian Sea)	RM3_1356	LR535943	Furfaro and Mariottini 2019
	Circeo Promontory, Latium (Tyrrhenian Sea)	RM3_1408	LR535944	Furfaro and Mariottini 2019
	W Andalusia, Spain (Atlantic Ocean)	BAU2952	LT718575	Furfaro *et al*., 2018
	W Andalusia, Spain (Atlantic Ocean)	BAU2953	LT718576	Furfaro *et al*., 2018

fragment and to exclude contaminations. Consensus sequence was aligned together with GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) sequence using the Muscle algorithm implemented in MEGA 6.0 (Tamura *et al*., 2013). Since the COI mitochondrial marker is the most commonly used for species barcoding in Heterobranchia, our analysed data set consisted of our one sequence and six sequences obtained from GenBank (Table 1). The number of COI base differences per site from averaging over the sequence pair was calculated, and the mean uncorrected *p*-distances were obtained using MEGA 6.0 software (Tamura *et al*., 2013).

RESULTS

During field observations, specimens of *P. soussi* could be found (from February to June up to 6-8 individuals could be found per dive) crawling on hard substrates covered by red algae and epiphytic hydrozoans. In young individuals, the notum was transparent so that the major internal organs, especially the reproductive organs, the hepato-pancreatic endings in the cerata and the cnidosacs, were visible (Fig. 2). In subadult and adult individuals, the red colour of the cerata with the typical white speckling, the whitish cnidosacs, the bluish colour of the base of the rhinophores and the white speckling of their terminal half, as well as the basal blue-purple and the white spotted apical part of cephalic tentacles, were clearly visible (Fig. 2). Among them, a single individual showed a different chromatic pattern, almost completely lacking any blue-violet coloration (Fig. 3). This specimen, here defined as a 'white morph', was recorded together with the typical *P. soussi* morphotypes here defined as 'typical morphs' (Furfaro *et al*., 2018; Furfaro and Mariottini, 2019). This adult individual, of about 10 mm in length, displayed nine rows of red orange cerata, densely spotted with white dots, notum, rhinophores and cephalic tentacles with white evident speckles (Fig. 3). Furthermore, the cerata apical part contained the cnidosacs, which were clearly visible through the transparent epithelium and which had a typical white ring in the upper part (Fig. 4A). The white pigment that covered the whole body reached the cephalic portion where the faint purple-violet masticatory plates were visible due to transparency (Fig. 4C).

Molecular analysis

DNA extraction and sequencing allowed to obtain a COI sequence 605 bp long (COI Accession Number: OQ921890). The results from the pairwise comparison revealed a range of 0.5% and 0.8% of uncorrected COI *p*-distances that are shown in Table 2 and confirmed the 'white morph' identification as *P. soussi*, even though it had an unusual body colour pattern.

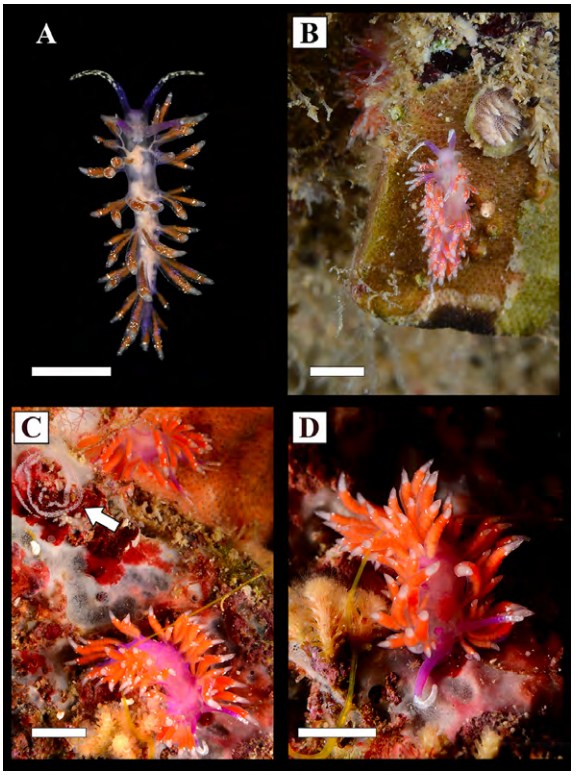

Fig. 2. Young (**A**), subadult (**B**) and adult (**C, D**) individuals of *Piseinotecus soussi* photographed in laboratory and *in situ* from Santa Maria al Bagno (Lecce, Italy, Ionian Sea). The typical egg ribbon (white arrow) is visible in (C). Scale bars = 5 mm.

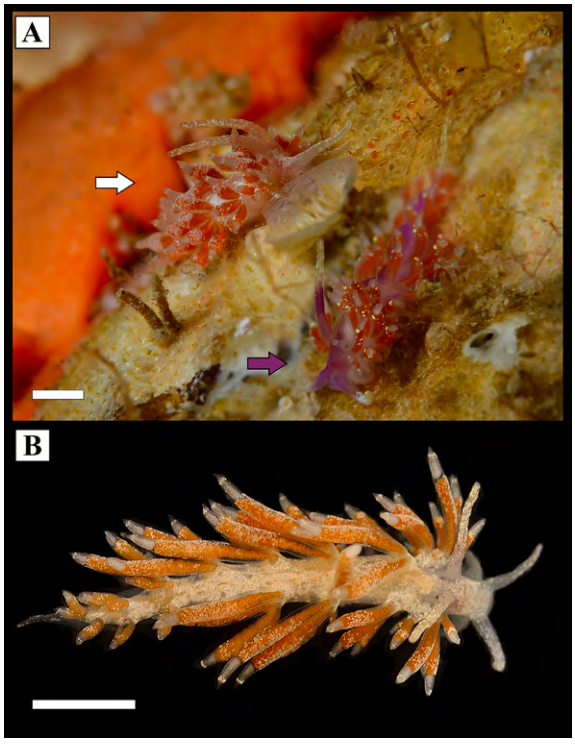

Fig. 3. Adult individuals of *Piseinotecus soussi*: the 'white morph' (white arrow) and a typical phenotype (violet arrow) photographed *in situ* from Santa Maria al Bagno (Lecce, Italy, Ionian Sea) (**A**); the 'white morph' individual (Voucher RM3_2262) photographed in laboratory (**B**). Scale bars = 2 mm.

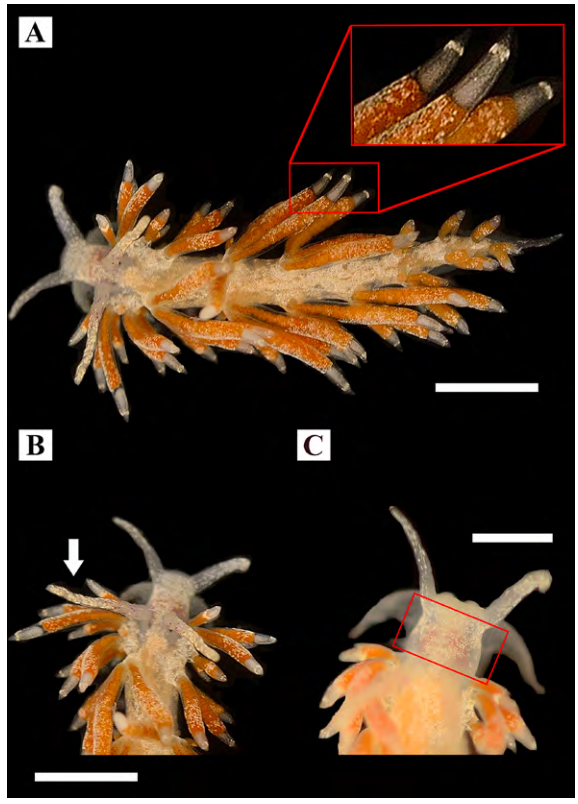

Fig. 4. *Piseinotecus soussi* 'white morph' details of the cerata (**A**); the smooth rhinophores (white arrow, **B**) and the cephalic portion (red frame) with the red masticatory plates visible through the epithelium (**C**). Scale bars 2 mm in A and B, and 1 mm in C.

DISCUSSION

The DNA identification method is considered a powerful tool for detecting hidden diversity and/or determining the ranges of intraspecific morphological variability within species. Here we report the case of DNA identification technique applied to a peculiar eolid nudibranch observed in Santa Maria al Bagno, in the Salento peninsula (Ionian Sea), which was initially identified as *Piseinotecus* sp. This specimen, named 'white morph' as the consequence of its overall white body, was found with *P. soussi* specimens, a rare species with a wide geographical range but poorly recorded in the Mediterranean Sea (Furfaro and Mariottini, 2019). To understand if the 'white morph' was conspecific with *P. soussi* or if it was perhaps a new Mediterranean species, DNA comparison was carried out using the COI mitochondrial molecular marker, the most used in nudibranchs. The COI sequence obtained from the 'white morph' specimen was compared with the ones already available in GenBank and belonging to *P. soussi*, revealing a range of *p*-distance between 0.5% and 0.8%, that is within the range of intraspecific variability widely

Table 2. Pairwise uncorrected *p*-distances (%) between *Piseinotecus soussi* specimens. Voucher numbers are given in Table 1. The 'white morph' specimen obtained in this study is RM3_2262.

	RM3_2262	RM3_1408	RM3_1356	RM3_863	RM3_862	BAU2953	BAU2952
RM3_2262	-						
RM3_1408	0.8	-					
RM3_1356	0.5	0.3	-				
RM3_863	0.7	0.5	0.2	-			
RM3_862	0.7	0.5	0.2	0.3	-		
BAU2953	0.5	0.3	0	0.2	0.2	-	
BAU2952	0.5	0.3	0	0.2	0.2	0	-

accepted for Nudibranchia molluscs (Furfaro *et al*., 2018; Furfaro and Mariottini 2021; Furfaro *et al*., 2021; Furfaro *et al*., 2022). Therefore, the obtained specimen was molecularly identified as *P. soussi* with distinctive body coloration, demonstrating that the combination of morphological observation and DNA identification is the best approach to resolve difficult taxonomic issues and to avoid misidentifications. This is particularly true in the case of Heterobranchia species, which are known to be morphologically very plastic in some cases (De Oliveira Saad *et al*., 2014). It is noteworthy to remind that *P. soussi* is apparently very similar to both *Edmundsella albomaculata* and *E. pedata*, so that it could be of particular interest to deepen the study of the chromatic pattern also in these two *Edmundsella* species, in order to assess if there are some rare and hidden atypical morphotypes also in these pink eolids. Finally, this study confirms the importance of avoiding taxonomic decisions based only on morphology and re-evaluating them by considering additional evidence using different approaches such as molecular, ecological, and chemical.

AUTHOR'S CONTRIBUTION

G.F., F.V., P.M., Conceptualization; G.F., F.V., C.L., A.T., Data curation; F.V., C.L., Formal analysis; F.V., C.L., Investigation; G.F., F.V., C.L., P.M., Methodology; P.M., Supervision; G.F., F.V., C.L., A.T., P.M. Roles/Writing - original draft; G.F., A.T., P.M., Writing - review & editing

ACKNOWLEDGMENTS

G.F. wishes to thank the 'MoSSHa - Monitoring of Species Sites and Habitats' project funded by the Apulian Region, within the POR Puglia 2014/2020 funds for monitoring actions of 'Natura 2000' species, sites and habitats.

REFERENCES

Aljanabi, S.M., Martinez, I. 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Research, 25(22), 4692-4693. https://doi.org/10.1093/nar/25.22.4692

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. Journal of Molecular Biology, 215, 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

Belmonte, G., Costantini, A., Sorrentino, F., Licchelli, C., Poto, M., Onorato, R. 2010. Le Grotte Sommerse dell'Area Maria Protetta "Porto Cesareo". Thalassia Salentina, 33(1), 15-38. https://doi.org/10.1285/i15910725v33p15

De Oliveira Saad, L., Cunha, C.M., Colpo, K.D., Valdés, A. 2014. It's not what it looks like: molecular data fails to substantiate morphological differences in two sea hares (Mollusca, Heterobranchia, Aplysiidae) from southern Brazil. Helgoland Marine Research, 68, 523-530. https://doi.org/10.1007/s10152-014-0408-1

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3, 294-299.

Furfaro, G., Mariottini, P. 2016. Check-list of the Nudibranchs (Mollusca Gastropoda) from the biodiversity hot spot "Scoglio del Corallo" (Argentario promontory, Tuscany). Biodiversity Journal, 7, 67-78.

Furfaro, G., Mariottini, P. 2019. Less rare than we thought: two new localities for *Piseinotecus soussi* Tamsouri, Carmona, Moukrim and Cervera, 2014 along the Tyrrhenian coast. Turkish Journal of Zoology, 43, 1-3. https://doi.org/10.3906/zoo-1809-18.

Furfaro, G., Mariottini, P. 2021. *Nemesignis*, a replacement name for *Nemesis* Furfaro and Mariottini, 2021 (Mollusca, Gastropoda, Myrrhinidae), preoccupied by *Nemesis Risso*, 1826 (Crustacea, Copepoda). Life, 11(8), 809. https://doi.org/10.3390/life11080809.

Furfaro, G., Picton, B., Martynov, A., Mariottini, P. 2016. *Diaphorodoris alba* Portmann and Sandmeier, 1960 is a valid species: molecular and morphological comparison with *D. luteocincta* (M. Sars, 1870) (Gastropoda: Nudibranchia). Zootaxa, 4193(2), 304-316. https://doi.org/10.11646/zootaxa.4193.2.6

Furfaro, G., Salvi, D., Mancini, E., Mariottini, P. 2018. A multilocus view on Mediterranean aeolid nudibranchs (Mollusca): systematics and cryptic diversity of Flabellinidae and Piseinotecidae. Molecular Phylogenetic and Evolution, 118, 13-22. https://doi.org/10.1016/j.ympev.2017.09.001

Furfaro, G., Salvi, D., Trainito, E., Vitale, F., Mariottini, P. 2021. When morphology does not match phylogeny: The puzzling case of two sibling nudibranchs (Gastropoda). Zoologica Scripta, 50, 439-454. https://doi.org/10.1111/zsc.12484

Furfaro, G., Schreier, C., Trainito, E., Pontes, M., Madrenas, E., Girard, P., Mariottini, P. 2022. The sea slug *Doriopsilla areolata* Bergh, 1880 (Mollusca, Gastropoda) in the Mediterranean Sea: another case of cryptic diversity. Diversity, 14(4), 297. https://doi.org/10.3390/d14040297.

Furfaro, G., Vitale, F., Licchelli, C., Mariottini, P. 2020. Two seas for one great diversity: Checklist of the marine Heterobranchia (Mollusca; Gastropoda) from the Salento peninsula (South-East Italy). Diversity, 12, 171. https://doi.org/10.3390/d12050171

Onorato, M., Belmonte, G. 2017. Submarine caves of the Salento peninsula: Faunal aspects. Thalassia Salentina, 39, 47-72. https://doi.org/10.1285/i15910725v39p47

Salvador, X., Fernández-Vilert, R., Moles, J. 2022. Sea slug night fever: 39 new records of elusive heterobranchs in the western Mediterranean (Mollusca: Gastropoda). Journal of Natural History, 56(5-8), 265-310. https://doi.org/10.1080/00222933.2022.2040630

Staden, R., Beal, K.F., Bonfield, J.K. 2000. The Staden package, 1998. *In* Bioinformatics Methods and Protocols. Methods in Molecular Biology, vol 132. (eds S. Misener, S. A. Krawetz). Humana Press, pp 115-130. https://doi.org/10.1385/1-59259-192-2:115

Tamsouri, N., Carmona, L., Moukrim, A., Cervera, J.L. 2014. Addenda to the article Bull Mar Sci. 90, 991-997 (2014): description of a new species of *Piseinotecus* (Gastropoda, Heterobranchia, Piseinotecidae) from the northeastern

Atlantic Ocean. Bulletin of Marine Science, 91, 83-84. https://doi.org/10.5343/bms.2014.1019.1

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30, 2725-2729. https://doi.org/10.1093/molbev/mst197

Zenetos, A., Mačić, V., Jaklin, A., Lipej, L., Poursanidis, D., Cattaneo-Vietti, R., Beqiraj, S., Betti, F., Poloniato, D., Kashta, L. 2016. Adriatic 'opisthobranchs' (Gastropoda, Heterobranchia): shedding light on biodiversity issues. Marine Ecology, 37, 1239-1255. https://doi.org/10.1111/maec.12306