Biodiversity and chemical ecology of selected gastropods from Iran, Persian Gulf

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Declaration

I hereby declare that I am the sole author of this thesis and that no other sources or learning aids, other than the ones listed, have been used. Furthermore, I declare that I have acknowledged the work of others by providing detailed references of said work.

Hereby I also declare that this thesis has not been prepared for another examination or assignment, neither wholly nor partially.

Erklärung

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Summary

Marine gastropods exhibit unique adaptations to marine environments have become a subject of interest in evolutionary biology and ecology. The marine slug genus *Peronia* J. Fleming, 1822, distinguished within the family Onchidiidae Rafinesque, 1815 by its dorsal gills, currently recognizes nine species. This study introduces a new species, *Peronia persiae* Maniei, Espeland, Mohavedi & Wägele, 2020, discovered in the Persian Gulf. This subtropical region characterized by challenging environmental conditions such as high salinity and temperature compared to the Indian Ocean. The new species was described based on comprehensive analyses involving molecular, histological, anatomical, micro-computer tomography, and scanning electron microscopy data. The distinction of the new species has been confirmed through the analysis of 16S ribosomal DNA (rDNA) and Cytochrome Oxidase I (COI) sequences using Automatic Barcode Gap Discovery (ABGD), the Generalized Mixed Yule Coalescent (GMYC), and the Bayesian Poisson Tree Processes (bPTP) methods.

To understand the chemical diversity within the genus *Peronia*, 12 individuals of the newly discovered *P. persiae*, along with a specimen of *P. verruculata* (Cuvier, 1830) from Bangka Island, Indonesia, were subjected to phylogenetic and chemotaxonomic analyses. Haplotype networking and species delimitation test resulted in the identification of nine well-supported clades and significant separation between *P. persiae* specimens and other clades. Metabolomic analysis using tandem mass spectrometry-based GNPS molecular networking unveiled a wide range of chemical diversity. While *P. persiae* specimens from different localities in the Persian Gulf exhibited a highly similar metabolome, only a few chemical features were shared across the clades with the *P. verruculata* specimen from Bangka Island, Indonesia. Polypropionate esters of onchitriols, ilikonapyrones, and osmoprotectant amino acid-betaine compounds were identified as the main common metabolites in both *Peronia* species. *P. persiae* exclusively contained isoflavonoids genistein and daidzein, whereas cholesterol and conjugated chenodeoxycholic acids were unique to *P. verruculata*. The presence of flavonoids, bile acids, and amino acid-betaine compounds in Onchidiidae, some of which are novel for panpulmonates, expands our knowledge of the chemical profiles in these organisms.

Another sea slug family in the northern coast of the Persian Gulf, Dendrodorididae O'Donoghue, 1924, globally inhabits intertidal zones with three genera including *Dendrodoris* Ehrenberg, 1831, *Doriopsilla* Bergh, 1880, and *Cariopsilla* Ortea & Espinosa, 2006. Here, three Dendrodorididae species were studied: *Dendrodoris fumata* (Rüppell & Leuckart, 1830), *Dendodoris nigra* (W. Stimpson, 1855), and a new *Doriopsilla* species. The study aimed to elucidate the relationships and differentiation among these species through a comprehensive molecular, anatomical, and histological data analyses. Anaylsis of 16S rDNA and COI sequences revealed that the genus *Dendrodoris* is paraphyletic. Within the *Dendrodoris* species, two clearly distant groups have been observed. Morover, the study confirmed the differentiation between *D. fumata* and *Dendrodoris rubra* (Kelaart, 1858). A new *Doriopsilla* species from the Persian Gulf was identified and supported by haplotype networking, genetic distance, and ABGD analyses of mitochondrial genes.

In short, our study on the biodiversity and chemical ecology of selected gastropods from Iran unveiled a new species within the genus *Peronia*, *P. persiae*, in the Persian Gulf. We provided detailed morphological descriptions supported by molecular and microscopic analyses. Chemical profiling of *Peronia* species revealed a diverse array of metabolites, highlighting the presence of unique compounds and the chemotaxonomic relationships within the genus. Furthermore, this work explored the genetic relationships and differentiation among Dendrodorididae species in the Persian Gulf, shedding light on the presence of cryptic species and the paraphyly of the genus *Dendrodoris*. The identification of a new *Doriopsilla* species and the confirmation of taxonomic synonymy added to our understanding of the phylogenetic relationships within the family. These findings contribute to the broader knowledge of gastropod biodiversity and chemical ecology, specifically in the context of the Persian Gulf region.

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Chapter 1

General Introduction

Persian Gulf

The Persian Gulf is home to a fascinating and unique diversity of life. A semi-enclosed area connected to the Sea of Oman and the Indian Ocean through the narrow Strait of Hormuz. The area is considered part of the larger Indo-Pacific covering approximately 251,000 square kilometers (96,912 square miles) region (Abuhijjleh *et al*., 2021; Bayani 2016; Samimi-Namin *et al*., 2023). The Gulf stretches about 990 kilometers (615 miles) from the Shatt al-Arab river delta in the northwest to the Strait of Hormuz in the southeast (https://maritimeducation.com/). The average depth is around 50 meters (164 feet) and a maximum depth of approximately 110 meters (361 feet) in its central basin (https://maritimeducation.com/). The Iranian coast is generally deeper than the Arabian coast, with depths ranging from 30 meters (98 feet) to 70 meters (230 feet). This region is marked by a series of narrow coastal plains and the Zagros Mountain range, which influence the topography of the seabed (Fatemi and Shokri 2001). Persian Gulf is subject to extreme environmental conditions due to its high latitudinal position, high evaporation rates, relative shallowness, coastline alterations, heavy maritime traffic, frequent oil spills, excessive industrial effluents, and restricted freshwater flow into the Gulf (Bayani 2016). Due to the harsh environments and unique species composition, the Gulf marked by low species diversity but a high degree of endemism compared with the rest of the Indian Ocean (Samini- Namin *et al*., 2023). Marine organisms residing in the Persian Gulf survive close to their environmental tolerance limits (Naser 2014). The water temperature fluctuates between 15 °C to 36 °C, with a maximum recorded sea-surface temperature of 38 °C in the summer of 1998 (Bayani 2016; Paparella *et al*., 2019). A rapid drop in seawater temperature (about 10 °C) marks the transition from the warm to cold season between late November and December (Fatemi and Shokri 2001). The northern region of the Persian Gulf, with its relatively cooler waters compared to the southern parts, provides a unique environment for the occurrence of gastropod species (Bayani 2016; Naser 2014). The ecosystem is highly adapted to extreme environmental conditions, but recent studies have shown that it is not the maximum temperatures but rather the length of exposure that harms marine life (Paparella *et al*., 2019). The annual evaporation rates range

between 1.37 to 4.8 meters per year, resulting in high salinity >40 ppt, increasing to >50 ppt in the southern bays of open ocean conditions and exceeding 70 ppt in evaporative lagoons (Abuhijjleh *et al*., 2021) and ranges from 37 to 38 ppt in the entrance of the Gulf up to 38 to 41 ppt in the extreme northwest (Evans 1999). It can provide a suitable habitat for a diverse range of marine life, including fishes and various groups of other invertebrates (Samimi-Namin *et al*., 2023). The Eastern Islands which are situated in the Strait of Hormuz are greatly influenced by the less saline and nutrient-rich oceanic waters from the Indian Ocean, while the inner islands tolerate a more saline and less fertile condition prevailed in nearly entire region (Fatemi and Shokri 2001). Studies focusing on the intertidal molluscs inhabiting the Persian Gulf have been limited in number, even though our understanding of the fauna in the Persian Gulf and Oman Sea regions has considerably expanded. During the last two decades, 1,618 species were documented within the geographical span of the Persian Gulf, Gulf of Oman, and the eastern Arabian Sea extending eastward to Bombay (Mumbai) (Al-Khadri *et al*., 2019), This includes gastropods reported by Salehi *et al* (2015) on the inter-tidal gastropod fauna of Deylaman bay on the north-east coast of Persian Gulf. The increasing number of publications on the Gulf's biodiversity, which cover various groups of marine organisms, underscores the necessity for further exploration and conservation of this area.

Subclass Heterobranchia **Burmeister, 1837**

Heterobranchia constitute a taxonomic category encompassing marine gastropods that display a broad range of morphological traits and adaptations for their respective ecological roles (Varney *et al*., 2021). This group includes numerous families and species, such as Onchidiidae and Dendrodoridídae, which are the focus of our study. Heterobranchia occupy a wide range of ecological niches and can be found in a wide range of marine habitats, including rocky shores, coral reefs, seagrass meadows, and pelagic zones (Varney *et al*., 2021). They have been documented in all major oceans and seas around the world, highlighting their global distribution such as the Indo-Pacific (Gosliner *et al*., 2015; Valdés and Gosliner 2001), Atlantic (Gosliner *et al*., 2015), Mediterranean (Padula *et al*., 2014), and Southern Oceans (Nimbs *et al*., 2016).

They feed on a diverse range of marine immobile organisms, including algae, sponges, cnidarians, bryozoans, and tunicates. Moreover, they frequently incorporate the chemical compounds from their food sources into their own defense mechanisms (Karmeinski *et al*., 2021).

Gastropods in general have extremely varied feeding habits, including grazing, browsing, suspension feeding, scavenging, detritivory, and carnivory (Goodheart *et al*., 2017). These feeding habits contribute to nutrient cycling and maintaining ecological balance in marine ecosystems. Heterobranchia species serve as important herbivores, controlling algal populations and promoting the growth of other marine organisms (Gosliner *et al*., 2015). They also serve as prey for higher trophic levels, contributing to food webs and energy transfer. However, various factors, such as water temperature, salinity, currents, food availability, habitat destruction, pollution, climate change, and overexploitation exert influence over these distribution patterns (Gosliner *et al*., 2015).

Family Onchidiidae

The family Onchidiidae, commonly referred to as "onchidiids," are characterized by their small to medium-sized slugs that lack shells and possess a muscular foot adapted for locomotion on land and in water (Dayrat 2009). All onchidiids are truly marine and are widespread throughout the world (Goulding *et al.*, 2022). Onchidiidae comprising various species that exhibit unique morphological features and ecological adaptations. The geographic distributions of onchidiid species indicate that allopatric speciation played a key role in the diversification of several genera, especially *Onchidella* and *Peronia* based on Goulding *et al*., 2022 study. Onchidiids are characterized by a particular combination of features, including absence or reduction of shells, specialized respiratory adaptations, special habitat preferences, body shape, coloration, and genetic differences. The genus *Peronia* Fleming, 1822 includes all the onchidiid slugs with dorsal gills (Dayrat *et al*., 2020).

Onchidiidae species are predominantly found in intertidal and shallow marine habitats, including mangroves, rocky shores, mudflats, and estuaries (Goulding *et al.*, 2022; Maniei *et al*., 2020a). They can be found in diverse regions around the globe, spanning tropical and subtropical zones such as the Indo-Pacific, Caribbean, and the Mediterranean Sea (Dayrat *et al*., 2011).

These slugs have a specialized respiratory structure called the pallial lung, which enables them to breathe air. They are adapted to withstand fluctuations in environmental conditions and can survive in oxygen-poor environments (Dayrat *et al*., 2011). Onchidiidae species are capable of burrowing in soft sediments and can tolerate exposure to air during low tides. They are known to

graze on microalgae and detritus, contributing to nutrient cycling and the ecological dynamics of their habitats (Jensen 1996).

Since Dayrat (2009), an exhaustive taxonomic revision of the family has been initiated, using both molecular and morphological approaches. This has led to the description of new taxa, revisiting and clarifying the nomenclatural status of almost all species of Onchidiidae. The phylogeny of the family has been reconstructed, recognizing a total of 37 genera (https://www.marinespecies.org) and 67 valid onchidiid species worldwide (Fernández-Gutiérrez *et al*., 2023). Despite their ecological significance, the taxonomy and phylogeny of the family Onchidiidae are still being elucidated.

Family Dendrodorididae

The family Dendrodorididae members are nudibranchs with elaborate dorsal gills and vibrant coloration (Brodie 2004). Dendrodorididae species exhibit a diverse range of morphological features and ecological adaptations, making them an interesting group for scientific study.

Dendrodorididae species can be found in various marine habitats, including coral reefs, rocky shores, and sea grass meadows and distributed in different regions around the world, including the Indo-Pacific, the Caribbean, and the Mediterranean (Carmona *et al*., 2013).

A notable characteristic of Dendrodorididae species is their feeding behavior. The members of the family are commonly recognized for their adaptations in the foregut, which have evolved as a response to the absence of a radula. These adaptations are specialized for suctorial feeding on sponges (Hirose *et al*., 2014). Some species of Dendrodorididae have been observed to selectively feed on toxic sponges, acquiring and retaining the sponge toxins for their own defense against predators. This ecological adaptation highlights the intricate relationships between Dendrodorididae species and their prey.

Taxonomy and phylogeny within the family Dendrodorididae are still being explored. Recent studies have utilized molecular techniques to gain insights into the evolutionary relationships and species diversity within the family. These studies led to the discovery of new species and contributed to our understanding of the evolutionary history of Dendrodorididae (Nimbs and Smith 2023; Galià-Camps *et al*., 2022; Furfaro *et al*., 2023).

Diverse morphological features, distribution patterns, and ecological adaptations make the family Dendrodoridídae an intriguing subject of study. Further research is needed to refine their taxonomy and understand their phylogenetic relationships.

Chemical strategies of Heterobranchia

Beyond physical attributes of Heterobranchia, according to numerous studies, marine slugs are constantly exposed to relatively high concentrations of bacteria, viruses, and fungi, many of which are harmful. Hence, if they do not have different defense methods, they are very vulnerable to these threats. These different defense methods include changing colour, resizing, and producing mucus (Arast *et al*., 2022). Some of these organisms have evolved fascinating chemical strategies that play important roles in their ecological interactions. Some species possess toxic chemicals or produce defensive compounds derived from their diet or symbiotic associations (Valdés *et al*., 2006; Cimino and Ghiselin 1999), such as the Chromodorididae family within the Nudibranchia comprising over 100 species (Papu 2021). The presence of these toxic compounds acts as a deterrent to predators, protecting the nudibranchs from predation (Gosliner *et al*., 2018). Chemical communication is another essential aspect of chemical ecology in Heterobranchia. Chemical signals play a crucial role in mate recognition, territory defense, and social interactions. For instance the Onchidiidae family exhibits chemical communication behaviors, using chemical cues for courtship and reproductive processes (Dayrat 2009).

Chemical interactions influence interspecies interactions, community dynamics, and ecosystem functioning. For example, when another species consumes a toxic or chemically defended Heterobranchia species, they may experience negative effects such as illness or death which can reduce their population size and alter their behavior. These phenomena alter the feeding relationships and behavior of the predators and can have effects on predator populations and community structure. Thrfore, chemical cues released by Heterobranchia can attract or repel other organisms, influencing their foraging behavior and habitat selection (Avila *et al*., 2018). These chemical interactions shape the composition and functioning of marine ecosystems.

The study of chemical ecology within the subclass Heterobranchia provides valuable insights into the fascinating chemical strategies employed by these marine gastropods. Understanding the ecological implications of these chemical interactions contributes to our broader knowledge of

marine ecosystems and highlights the importance of chemical ecology in shaping the diversity and functioning of Heterobranchia species.

Scope of the present study

The aim of this study was to investigate the biodiversity and chemical ecology of some slug species, with a focus on the *P. verruculata* complex and the genera *Dendrodoris* and *Doriopsilla*, in the Persian Gulf, Iran. To achieve this, an integrative approach, combining morphological and molecular analyses as well partly chemical analyses was employed.

The first objective was to investigate the *P. verruculata* complex by conducting an extensive analysis of available sequences from literatures and the NCBI database. This analysis was supplemented with new sequences obtained from Iranian specimens specifically in this study. This analysis aimed to clarify the species identities and explore the relationships within the complex (Chapter 3).

Furthermore, our study pioneered a combined phylogenetic and metabolomic survey of slug species belonging to the genus *Peronia*. We specifically investigated the geographic relationship between Iranian *Peronia* haplotypes from different localities, utilizing phylogenetic reconstruction, species delimitation, and haplotype networking. Additionally, we compared the secretome of phylogeographically distinct species to that of other *Peronia* specimens widely distributed in the Indo-Pacific. This comparative analysis aimed to identify a potential core metabolome and discern species-specific traits (Chapter 4).

In the third investigation, we conducted an examination of specimens belonging to *D. nigra, D. fumata*, and an undiscovered *Doriopsilla* species located along the Iranian intertidal coastline of the Persian Gulf. Our primary approach involved histological methods and analysis of partial CO1 and 16S rRNA gene sequences, encompassing all existing sequences from these genera and incorporating previously published data from the Persian Gulf (referencing Fatemi *et al*., 2021). To ascertain species identities and delineate their relationships within the genera, we employed various analytical techniques (Chapter 5).

To addresse these aims morphological, molecular, phylogenetic, and metabolomic approaches were applied and provided valuable insights into the biodiversity and chemical ecology of selected slug species in the Persian Gulf. The discovery of a new species, along with its distinct

morphology, contributed to our understanding of *Peronia* species diversity, and also a new *Doriopsilla* species in the Persian Gulf, backed by haplotype networking, genetic distance, and ABGD analyses of mitochondrial genes. Also the paraphyly of the genus *Dendrodoris* was found despite the monophyly of our species *D. nigra* and *D. fumata*.

Chapter 2

Materials and Methods

Collection of specimens

Collection of *Peronia persiae* specimens (14 specimens) was conducted in the intertidal zone during low tide at Bandar Lengeh (26° 33′ 29″ N 54° 52′ 50″ E) and Lavan Island (26° 48′ 20.99″ N 53° 16′ 4.80″ E), in Iran in March 2015 and February 2016, respectively. Specimens were collected from the surface of rocks, some of them were found hidden in rock crevices. The specimens were photographed using a digital camera Canon SX160IS and measured for length. Temperature, pH value, and salinity were measured at Lavan Island in 2016. All specimens had a length of 4-6 cm and 12 of them were preserved in 96% ethanol and two of them used for histology studies. Additionally, one *P. verruculata* specimen preserved in 96% ethanol provided from Bangka Island (2° 15′ 0″ S, 106° 0′ 0″ E), Indonesia. Voucher specimens of *P. persiae* were deposited at the Zoologische Staatssammlung München, Germany. The specimen of *P. verruculata* which is a part of the reference collection of Sam Ratulangi University, Indonesia, was kindly provided by Dr. A. Papu.

Also a total of 12 specimens of *D. nigra*, 18 specimens of *D. fumata*, and 6 specimens of the newly discovered species *D. aroni* **sp. nov**. were collected again in the intertidal zone during low tide at Bandar Lengeh and Lavan Island in February to April 2015 and March 2016. Some of the animals were photographed alive using the digital camera. From each species, one sample was used for histology and the rest used for molecular studies. The collected material is deposited at the Leibniz Institute for the Analysis of Biodiversity Change - Zoological Museum Hamburg (ZMH).

Anatomical observation

Histology

Desired specimens, as well as the reproductive organs of selected individuals, were subjected to dehydration in EtOH and embedded in hydroxyethyl methacrylate (Heraeus Kulzer GmbH) for serial sectioning. Sections (2.5 μm) were stained with toluidine blue, photographed subsequently

under a ZEISS Microscope (Imager.Z2m) and analysed with ZEN software (ZEISS) at Alexander Koenig Research Museum in Bonn, Germany

Scanning electron microscopy

Dissection was performed under an Olympus SZX12 stereo microscope. Radula, penis, and needle of penial accessory gland of selected specimens were extracted by using a 5% KOH solution, dried and analysed using a Scanning Electron Microscope (ZEISS Sigma VP 300).

Micro-computer tomography

One specimen of *Peonia* was stained for two days in 1% iodine dissolved in 100% Ethanol (I2E). Reconstructed images were analysed in CTVox.

DNA extraction, amplification, and sequencing

DNA isolation was carried out using the Qiagen DNeasy Blood and Tissue kit, following manufacturer's instructions. Partial sequences of mitochondrial COI (ca. 680 bp) and ribosomal 16S (ca. 550 bp) were amplified by polymerase chain reaction (PCR) using the primers LCOI490-JJ (5'-CHACWAAYCATAAAGATATYGG-3') and HCO2198-JJ (5'-AWACTTCVGGRTGVCCAAARAATCA-3') (Astrin & Stüben 2008) for COI; 16Sar-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr-H (5'- CCGGTCTGAACTCAGATCACGT-3') (Palumbi *et al*., 1991). The thermoprofile during the PCR used for COI and 16S was: 15 min at 95°C; 40 cycles of reaction conditions (48 cycles for 16S) involved an initial denaturation of 94°C for 35sec (45 sec for 16S), subsequent annealing at 55°C for 90 sec (56°C for 45 sec for 16S), elongation at 72°C for 90 sec and final elongation step of 72°C for 10 min. Sequencing was performed by Macrogen Europe (Amsterdam, Netherlands).

Phylogenetic reconstruction

Sequences were edited using BioEdit (ver.7.2.6.1) (Hall 1999) and aligned using MAFFT (Katoh *et al*., 2002) in Geneious v7.1.9 (Kearse *et al*., 2012). After trimming, the alignments of the mitochondrial genes comprised for 16S, CO1 and the concatenated dataset. The alignments of the nuclear genes comprised for 18S and H3 just for Dendrodoridiidae family. Maximum likelihood (ML) analyses were run in IQ-TREE (Nguyen *et al*., 2014; Trifinopoulos *et al*., 2016) using the online version 1.6.3 on a webserver (http://iqtree.cibiv.univie.ac.at/), with the GTR model for all

gene and gene sets (CO1, 16S and concatenated data, as well as the 18S and H3 data set). Support values were calculated based on 1000 ultrafast bootstrap replicates and the approximate likelihood ratio test (SH-aLRT) (2000 replicates). Dendroscope (version 3.5.8) (Huson & Scornavacca 2017) and Inkscape (version 0.92) (https://inkscape.org/en/) were used to edit the phylograms. More details for the various methods are provided in the specific chapters.

Species delimitation test

In general three different methods were used for delimiting species: Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al*., 2011), General Mixed Yule Coalescent model (GMYC) (Monaghan *et al*., 2009; Pons *et al*., 2006), and Poisson Tree Processes (bPTP) (Zhang *et al*., 2013). ABGD was applied separately to COI and 16S datasets, as well as the concatenated dataset, using default values under the Kimura, K80 model. Ultrametric trees were generated for GMYC using penalized likelihood (Sanderson 2002), and the best clock model was selected based on the phi information criterion (Paradis 2013). GMYC analyses were performed using the SPLITS package (Ezard *et al*., 2009). bPTP analyses were run on COI and 16S trees and uploaded to the PTP web server (http://species.h-its.org/) (Zhang *et al*., 2013). COI minimum and maximum pairwise uncorrected p-distances, between and within the main clades, were calculated using Species Identifier (Meier *et al*., 2006). More details are provided in the specific chapters.

Haplotype network

A statistical parsimony analysis (Templeton *et al*., 1992) was performed using the program TCSv.1.21 (Clement *et al*., 2000) in PopART (Liegh *et al*., 2015). The analysis identified shared haplotypes among individuals and calculated the number of substitutions connecting the haplotypes in the network. The program also used for the inclusion and visualization of geographic information within the network. The settings used were a 95% connection limit and 5,000 iterations. The haplotype analysis also incorporated available geographic information for each sequence.

Chemical analyses

Metabolite extraction and HPLC–MS/MS analysis

The alcohol of samples container was evaporated under vacuum, re-dissolved in methanol, and analyzed using a micrOTOF-QIII mass spectrometer coupled with an HPLC Dionex Ultimate 3000 at the Institute for Pharmaceutical Biology, University of Bonn.

Molecular networking

The MS/MS data from different groups were converted to mzXML format and submitted to the GNPS server. A molecular network was generated by merging identical spectra into nodes representing parent masses and connecting compounds with similar fragmentation patterns. Filtering was applied to remove MS/MS peaks within ±17 Da of the precursor m/z, and only the top 6 peaks within a ±50 Da window were selected. MS-Cluster was used to cluster the resulting data based on a parent mass tolerance of 0.02 Da and an MS/MS fragment ion tolerance of 0.02 Da, resulting in consensus spectra. Networks were created with edges filtered based on cosine scores above 0.5 and more than 4 matched peaks. The network spectra were searched against GNPS spectral libraries, and matches with scores above 0.5 and at least four matched peaks were retained. In silico identification of natural products was performed using DEREPLICATOR plus. The network was visualized using Cytoscape 3.6.1, and the molecular network file is available on the NDEx site.

Chapter 3

Description of a new *Peronia* species (Gastropoda: Eupulmonata: Onchidiidae) from Iran, Persian Gulf

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Abstract

Peronia J. Fleming, 1822 is a eupulmonate slug genus with a wide distribution in the Indo-Pacific Ocean. At the moment nine species are considered as valid. However, molecular data indicate cryptic speciation and more species involved. Here, we present results on a new species found in the Persian Gulf, a subtropical region with harsh conditions such as elevated salinity and high temperature compared to the Indian Ocean. *Peronia persiae* **sp. nov.** is described based on molecular, histological, anatomical, micro-computer tomography and scanning electron microscopy data. ABGD, GMYC and bPTP analyses based on 16S rDNA and cytochrome oxidase I (COI) sequences of *Peronia* confirm the delimitation of the new species. Moreover, our 14 specimens were carefully compared with available information of other described *Peronia* species. *Peronia persiae* **sp. nov**. is distinct in a combination of characters, including differences in the genital (ampulla, prostate, penial hooks, penial needle) and digestive systems (lack of pharyngeal wall teeth, tooth shape in radula, intestine of type II)

Key words: Histology, species delimitation, phylogeny, *Peronia verruculata*, Systellommatophora, Mollusca

Introduction

The usually shallow Persian Gulf (average depth 50 m), located in the subtropical northwest of the Indian Ocean, is exposed to harsh conditions and limited exchange of water through the Strait of Hormuz. Sea surface temperature range from 24 to 32°C in the Strait of Hormuz and 16 to 32°C in the extreme northwest, with a short cold spring and a long warm summer seasons (Amini Yekta *et al*. 2013). Seawater salinity ranges from 37 to 38 ppt in the entrance of the Gulf up to 38 to 41 ppt in the extreme northwest, and the range of the tides varies according to the locality from 1.4 m up to 3.2 m (Evans 1999). This contrasts the typical water conditions of the Indian Ocean, with sea surface temperature ranges of 22.2 to 27.7°C (Sawe 2018), and a lower salinity range of 36.5−37.2 ppt (Pous *et al*. 2015). Therefore, the Persian Gulf differs from the Indian Ocean regarding these physiochemical parameters and an impact on the diversity and ecology especially of the intertidal fauna is very likely. However, almost no biodiversity studies exist from this region. Only a few ones documented the occurrence of marine Heterobranchia along the south Iranian shorelines (Amini Yekta *et al*. 2012; Amini Yekta *et al*. 2013; Attaran *et al.* 2015, Rezai *et al.* 2016), with a single species of the eupulmonate Onchidiidae recorded: *Peronia peronii*

(Cuvier, 1804), identified either by external morphology (Amini Yekta *et al*. 2012) or by 18S rDNA sequences in comparison to other heterobranchs (Attaran *et al.* 2015).

Members of the Onchidiidae, a family within the eupulmonate group Systellommatophora, have a widespread distribution and are quite common in the middle and upper intertidal zone, either in rocky, muddy or sandy habitats (Dayrat 2009; Zhang *et al*. 2016). Most species are exclusively found in tropical and subtropical areas of the Indo-West Pacific (Wu *et al*. 2010). Onchidiids have no shell and the notum can be smooth or covered with tubercles in different sizes and shapes. Several characters are typical for certain genera, others rather unique. Multiple photoreceptors including dorsal eyes on the notum, for example, are characteristic for the genera *Onchidium* Buchannan, 1800, *Peronina* Plate, 1893, *Melayonchis* Dayrat & Goulding, 2017, *Wallaconchis* Goulding & Dayrat, 2018, *Alionchis* Goulding & Dayrat, 2018, and *Marmaronchis* Dayrat & Goulding, 2018, but are absent in *Onchidella* J.E. Gray, 1850, and *Onchidina* Semper, 1882. Branchial gills on the notum are typical for *Peronia*. In former times, they were also reported for *Paraperonia* Starobogatov, 1976 (now accepted as synonym of *Peronia*, Dayrat *et al*., 2020) and *Scaphis* Starobogatov, 1976.

Onchidiidae have been recognized since 1815 as a natural taxon and its monophyly was confirmed in recent studies (Dayrat 2009; Dayrat *et al*. 2011). However, the relationships of genera and between species remain poorly understood. Furthermore, species identification is hampered by the lack of distinct external features. Although the genus *Peronia* has nine currently accepted species (MolluscaBase 2018) and is one of the oldest described onchidiid genera, only a few additional studies besides species original descriptions are available. *Peronia verruculata* (Cuvier 1830) was thoroughly investigated by Awati & Karandikar (1948) using histological methods, but unfortunately, they did not mention the locality of the specimens they investigated. The type locality of *P. verruculata* is mentioned with India, Bandra, and the species seems to have a very wide distribution in the Indo-Pacific Ocean (GBIF, 2019). Further information for this species was provided by Plate (1893), Labbé (1934), Britton (1984) and Hyman (1999). *P. peronii* was re-investigated only a few times (Labbé 1934; Plate 1893) and additional information on the other seven described species is rarely available. Synonymization of various genera with *Peronia* has rather added to the confusion surrounding this clade, for example *Quoyella* Starobogatov, 1976 and its monotypic species *Q. indica* Labbé, 1934 (now *Peronia indica*). In this case, the possession of branchial gills led to the synonymization with *Peronia*; while other

morphological characters i.e. two separate male openings and intestinal type five would not support the species in the genus *Peronia* (Table S1).

In this study, we address the *P. verruculata* complex by re-analysing all available literature data, and analysing all available sequences from NCBI together with new sequences from Iranian specimens. We describe a new species from the Persian Gulf based on an integrative morphological and molecular approach and show its distinctiveness in morphology from all other valid *Peronia* species, including the close related species *P. verruculata*.

Material and Methods

Sampling was undertaken in the intertidal zone during low tide at Bandar Lengeh (26°33′29″N 54°52′50″E) in March 2015, and Lavan Island (26°48′20.99″N 53°16′4.80″E) in February 2016 (Figure 1). In total, 14 specimens of *Peronia* sp. were collected in the intertidal zone from the surface of rocks. Some of them were hidden in rock crevices but could be traced by the trails produced during feeding. The animals were photographed alive using a digital camera (Canon SX160IS) and measured for length. For details of locality and preservation method see Table 1. Temperature, pH value and salinity were measured at Lavan Island in 2016.

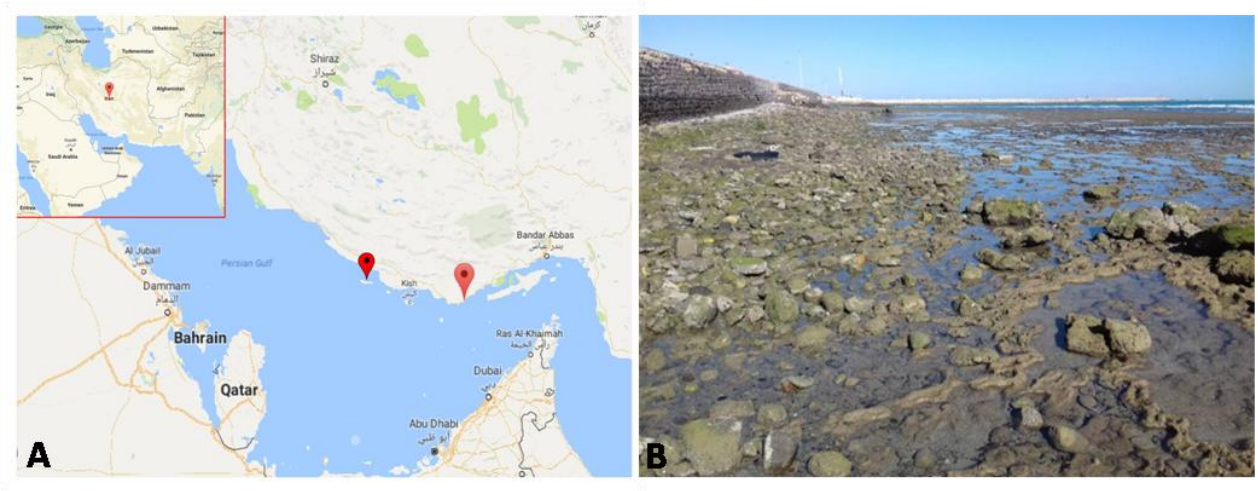

FIGURE 1. Information about collecting sites of *Peronia persiae* **sp. nov**.: A. Localities of collection sites. B. Typical rocky shore exposed during low tides.

Anatomical observation. Dissection was performed under an Olympus SZX12 stereo microscope. Radula, penis, and needle of penial accessory gland were extracted by using a 5%

KOH solution, dried and analyzed using a Scanning Electron Microscope (ZEISS Sigma VP 300). For Micro-CT analyses one specimen was stained for two days in 1% iodine dissolved in 100% Ethanol (I2E). Reconstructed images were analysed in CTVox. For histological analyses, two specimens and the genital system of an additional specimen were dehydrated in EtOH and embedded in hydroxyethyl methacrylate (Heraeus Kulzer GmbH) for serial sectioning. Sections (2.5 µm) were stained with toluidine blue, photographed subsequently under a ZEISS Microscope (Imager.Z2m) and analysed with ZEN software (ZEISS) at Alexander Koenig Research Museum in Bonn, Germany.

DNA extraction, PCR, and DNA sequencing. DNA isolation was carried out using the Qiagen DNeasy Blood and Tissue kit, following manufacturer's instructions. Partial sequences of mitochondrial COI (ca. 680 bp) and ribosomal 16S (ca. 550 bp) were amplified by polymerase chain reaction (PCR) using the primers LCOI490-JJ (5' –CHACWAAYCATAAAGATATYGG-3') and HCO2198-JJ (5'-AWACTTCVGGRTGVCCAAARAATCA-3') (Astrin & Stüben 2008) for COI; 16Sar-L (5′ -CGCCTGTTTATCAAAAACAT-3') and 16Sbr-H (5′-CCGGTCTGAACTCAGATCACGT-3') (Palumbi *et al*. 1991). The thermoprofile during the PCR used for COI and 16S was: 15 min at 95 °C; 40 cycles of reaction conditions (48 cycles for 16S) involved an initial denaturation of 94 °C for 35sec (45 sec for 16S), subsequent annealing at 55 °C for 90 sec (56 °C for 45 sec for 16S), elongation at 72 °C for 90 sec and final elongation step of 72 °C for 10 min. Sequencing was performed by Macrogen Europe (Amsterdam, Netherlands). Sequences are deposited in GenBank with the accession numbers listed in Table 2.

Phylogenetic reconstruction. Sequences were edited using BioEdit (ver.7.2.6.1) (Hall 1999) and aligned using MAFFT (Katoh *et al*. 2002) in Geneious v7.1.9 (Kearse *et al*. 2012). Sequences of other species from all available genera of the family Onchidiidae (446 sequences) and outgroups (three sequences) were downloaded from GenBank, from Wang *et al*. (2009); Chen *et al* (2011); Dayrat *et al*. (2011, 2014, 2016, 2017, 2018, 2019); Dayrat & Goulding (2017); Cumming *et al* (2014); Goulding *et al.* (2018a, 2018b, 2018c); Layton *et al*. (2014) (Table 2). All sequences were included in the phylogenetic analysis to evaluate the correct affiliation and monophyly of *Peronia* for subsequent analyses. Two species of *Siphonaria* G. B. Sowerby I, 1823 (*S. zelandica* Quoy & Gaimard, 1833 and *S. sirius* Pilsbry, 1894), considered as the closest relatives to the Onchidiidae (Wägele *et al*. 2014) were included as outgroup for rooting the tree.

TABLE 1. Specimens used in this study. Abbreviations: BL = Bandar Lengeh; LA = Lavan Island.

Specimen	Preservation	Purpose	Length of preserved animal (mm)
BL 1	ETOH 96%	Paratype; DNA barcoding, SEM, dissection	37
BL 2	Formalin	Histology	32
LA 1	ETOH 96%	Paratype; DNA barcoding, SEM, dissection	32
LA 2	ETOH 96%	Paratype; DNA barcoding, SEM, dissection	22
LA 3	ETOH 96%	Voucher; DNA barcoding, SEM, dissection	26
LA4	ETOH 96%	Voucher; DNA barcoding, SEM, dissection	28
LA 5	ETOH 96%	Voucher; DNA barcoding, SEM, dissection	34
LA 6	ETOH 96%	Voucher; DNA barcoding, SEM, dissection	32
LA 7	ETOH 96%	**Holotype**; DNA barcoding, SEM, dissection of head area	35
LA 8	ETOH 96%	SEM, DNA barcoding, dissection	25
LA9	ETOH 96%	SEM, DNA barcoding, dissection	22
LA 10	ETOH 96%	SEM, DNA barcoding, dissection, Histology of genital system	13
LA 11	Formalin	Histology	29
LA 12	ETOH 96%	Micro CT, dissection	31

TABLE 2. Name of species, locality information, COI and 16S GenBank accession numbers for genus *Peronia*. # registered but unpublished data of specimens taken from NCBI.

Preliminary name taken from literature (NCBI)	New assigned / Confirmed name	Locality	GenBank COI	GenBank 16S
Scaphis sp.		Philippine Islands	HQ660050	HQ659918
Onchidium verrucosum	*Peronia verruculata* (misspelling by Göbbeler and Klussmann-Kolb 2011)	Australia (Queensland)	EF489391	EF489316
Peronia sp. 1		Hawaii	HQ660038	HQ659906
Peronia sp. 2		Oman	HQ660044	HQ659912
Peronia sp. 3		Queensland (Australia)	HQ660048	HQ659916
Peronia sp. 4		Mozambique	HQ660045	HQ659913
Peronia sp. 5		Mozambique	HQ660047	HQ659915
Peronia sp. 6		Indonesia (Sulawesi)	HQ660046	HQ659914
Peronia sp. 7		Hainan (China)	HQ285979	HQ285967
Peronia sp. 8		Hainan (China)	HQ285980	HQ285968
Peronia sp. 9		Hainan (China)	HQ285981	HQ285966
Peronia sp. (unpublished paper by Chen *et al.* 2011)	*Peronia verruculata* (reassigned by Sun *et al.* 2014)	Hainan (China) #	JN543165	JN543101
Peronia cf. *verruculata* (Dayrat *et al.* 2011)	*Peronia* sp. (reassigned by Dayrat *et al.*2016)	Okinawa (Japan)	HQ660043	HQ659911
Peronia peronii		Guam	HQ660041	HQ659909
Peronia cf. *peronii*		Mozambique	HQ660042	HQ659910
Peronia verruculata (unpublished paper by Wang *et al*. 2009)		Fujian (China) #	GU166568	-
		Fujian (China) #	GU166567	-
		Fujian (China) #	GU166566	GQ985284
		Fujian(China) #	GU166564	GQ985282
		Fujian(China) #	GU166563	GQ985281
		Hainan(China)#	GU166561	GQ985277
		Hainan(China) #	GU166559	GQ985275
		Hainan(China) #	GU166558	GQ985274
		Hainan(China) #	GU166562	GQ985278
		Hainan(China) #	GU166560	GQ985276
		Fujian(China) #	GU166565	GQ985283
		Hainan(China) #	GU166557	GQ985273
Peronia verruculata (unpublished paper by Chen *et al*. 2011)		Fujian (China) #	JN543154	JN543090
		Hainan (China) #	JN543153	JN543089
		Zhanjiang (China) #	JN543152	JN543088

Peronia sp.		Singapore	MH002607	-
			MH002590	-
			MH002605	-
			MH002603	-
			MH002600	-
			MH002599	-
			MH002594	-
			MH002591	-
			MH002589	-
			MH002586	-
			MH002585	-
			MH002580-MH002582	-
			MH002575-MH002578	-
			MH002592	-
			MH002596	-
			MK142731	-
			MK142730	-
			MK142725-MK142728	-
			MK142720-MK142723	-
			MK142717	-
			MK142715	-
			MK142714	-
			MK142712	-
			MK142704	-
			MK142706-MK142709	-
			MH002597	-
			MH002604	-
			MH002579	-
			MH002598	-
			MH002602	-
			MH002583	-
			MK142710	-
			MH002593	-
			MK142729	-
			MK142713	-

			MH002595	-
			MK142732	-
			MK142724	-
			MK142716	-
			MH002588	-
			MK142705	-
			MK142719	-
			MK142711	-
			MH002584	-
			MH002606	-
			MH002601	-
			MK142718	-
Peronia sp. 2		Singapore	MK142680-MK142703	-
			MH002574	-
			MH002573	-
			MH002569	-
			MH002567	-
			MH002566	-
			MH002559-MH002563	-
			MH002548-MH002556	-
			MH002546	-
			MH002545	-
			MH002571	-
			MH002572	-
			MH002547	-
			MK142694	-
			MK142689	-
			MH002564	-
			MH002550	-
			MH002557-MH002558	-
			MH002570	-
Peronia persiae **sp. nov.**		Bandar Lengeh	MK312166	MK312167
		Lavan Island	MK993386-MK993395	MK993398-MK993407
Peronia sp. 7		Indonesia, Bangka Island	MK993396	MK993397

After trimming, the alignments comprised 454 bp for 16S, 635 bp for COI and 1075 bp for the concatenated dataset. The maximum likelihood (ML) analysis was run in IQ-TREE (Nguyen *et al*. 2014; Trifinopoulos *et al*. 2016) based on the concatenated data set using the online version 1.6.3 on a webserver (http://iqtree.cibiv.univie.ac.at/), with the GTR model for each gene. Support values were calculated based on 1000 ultrafast bootstrap replicates and the approximate likelihood ratio test (SH-aLRT) (2000 replicates). Subsequent analyses were performed with a reduced and concatenated alignment, only including *Peronia* sequences (Table 2), with identical sequences removed. For this analysis, *Wallaconchis graniferus* (Semper, 1880) was chosen as outgroup, based on the results of the general Onchidiidae analysis. Species delimitation tests were performed with the same taxa, but with 16S and COI genes datasets separated, and the results presented on the COI (Figure 11) and the concatenated trees (Figure 12). Dendroscope (version 3.5.8) (Huson & Scornavacca 2017) and Inkscape (version 0.92) (https://inkscape.org/en/) were used to edit the phylograms.

Species delimitation. The Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.* 2011), General Mixed Yule Coalescent model (GMYC) (Monaghan *et al.* 2009; Pons *et al.* 2006) and Poisson Tree Processes (PTP) (Zhang *et al.* 2013) were applied for delimiting the species. ABGD is independent of predefined species groups (Puillandre *et al.* 2011; Padula *et al.* 2014) and was applied to COI and 16S datasets separately and as concatenated datasets, using default values under the Kimura, K80model. GMYC is a likelihood method for delimiting species by fitting within- and between-species branching models on phylogenetic trees (Pons *et al*. 2006; Monaghan *et al.* 2009). bPTP is an updated version of the original maximum likelihood PTP program (maximum likelihood PTP search result is part of the bPTP results) and adds Bayesian support (BS) values to delimited species on the input tree (Figures S1–S2) (Zhang *et al.* 2013).

Ultrametric starting trees for GMYC were generated using penalized likelihood (Sanderson 2002) using the chronos function in the APE package (Paradis *et al.* 2004) in R. Four clock models: strict, discrete rate variation with ten rate categories, correlated and uncorrelated-relaxed were fitted on the ML trees for COI and 16S using the chronos function in the APE package (Paradis *et al.* 2004). The best model was selected using the phi information criterion, which takes the penalized term into account (Paradis, 2013). All models were fitted for trees based on both COI and 16S with the smoothing parameter, lambda, set to 0.1, 0.5 and 1.0, and in each case, the strict clock was found to be the best model. Ultrametric trees for both genes based on a strict clock with lambda=1 were used in subsequent analyses. Single threshold GMYC analyses were

performed in R using the SPLITS package (Ezard *et al.* 2009). The PTP model assumes that the number of substitutions is significantly higher between species than within species, which is reflected in the branch lengths (Zhang *et al.* 2013). bPTP analyses were run on COI and 16S trees resulting from the ML analyses and uploaded, individually, as Nexus files to the PTP web server (http://species.h-its.org/) (Zhang *et al.* 2013). Trees were rooted and *W. graniferus* was included as outgroup. bPTP graphic results for each gene were presented as PhyloMaps (Zhang *et al.* 2011). COI minimum and maximum pairwise uncorrected p-distances, between and within the main clades, were calculated using Species Identifier (Meier *et al.* 2006).

Results

Systematics

Family Onchidiidae Rafinesque, 1815

Genus *Peronia* J. Fleming, 1822

Type species *Peronia peronii* (Cuvier, 1804)

Diagnosis. Presence of contractible gills distributed irregularly at least in the posterior part of the dorsal notum. Notum always narrower than the pedal sole; warty with dorsal eyes single or in groups. Some species characterized by spicules in the integument. Intestine usually of type I or type II. Rectal gland absent. Penis short with many spines. Penial gland very long with a large muscular sac. Needle apparatus in penial gland sac present (Labbé 1934; Britton 1984; Dayrat 2009).

***Peronia persiae* sp. nov. Maniei, Espeland, Movahedi & Wägele**

(Figs. 2 –10)

ZooBank LSID: urn:lsid:zoobank.org:pub:2F2B0734-03E2-4D94-A72D-9E43A132D1DE

Type material. Holotype: Zoologische Staatssammlung München (ZSM Mol 20180017), Lavan Island (26°48′20.99″N 53°16′4.80″E), collected in February 2016, 35 mm in length. Paratypes: Zoologische Staatssammlung München (ZSM Mol 20180018), Bandar Lengeh (26°33′29″N 54°52′50″E), collected in March 2015 (1 specimen), 32 mm in length; Lavan Island (26°48′20.99″N 53°16′4.80″E), collected in February 2016 (2 specimens), 22 and 37 mm in length.

Etymology. *Peronia persiae* **sp. nov.** is named after the home country of the first author, Iran (Persia).

External morphology (Figure 2). Living specimens with 20-65 mm in length, and 13-37 mm when preserved in formalin. Elongate while moving, but more oval to round in outline when resting. Notum of living animals muddy green to gray in colour and covered with tubercles (papilla) (Figure 2A-B). Foot elongate, smooth and light green; covered by hyponotum (Figure 2A). Eight to 16 tubercles bearing dorsal eyes in groups of two to four (Figure 2C). In posterior region of the mantle, six to 16 irregularly branched branchial gills (so called gill-trees) (Figure 2D). Gills expanding only when the animal is submerged. Two retractable tentacles with an apically lying eye between mantle and foot in the head region. Ventrally lying mouth surrounded by oral lips. Male genital pore between right tentacle and labial palp (Figure 2A). Anus located ventrally at the posterior end of the body in the middle between mantle and foot; female opening on the right side of the anus; pneumostome posterior to the anus and opening only when the slug is out of the water.

Integument. Epidermis composed of small cells; completely covered by a thin cuticular-like layer. Many bluish stained glandular cells present, reaching deep into the notum tissue; glandular cells staining violet (mucopolysaccharides) interspersed. Some cells without distinct contents (probably empty gland cells), also reaching into notum tissue (Figure 3A).

Vision system. Oval shaped stalk eye on the tip of each tentacle; consisting of cornea, lens and pigmented retina layer (Figure 3B). The latter comprising a villous, pigmented layer, a somatic layer and neural layer with nerve fibres. The dorsal eyes on the notum of inverse nature: the position of the retinal layer inverted with the main nerve penetrating the pigment layer surrounding the retina. Lens composed of two parts: Principal lens consisting of one cell with a large nucleus and thick microvilli layer facing towards epidermis. Accessory lens lying beneath principle lens; formed by at least one cell with a large nucleus. Epidermis covering dorsal eyes similar to surrounding epidermis, but with smaller cells above the eyes (Figure 3C). Photoreceptors as extraocular structures scattered beneath the epidermis, partly single or forming clusters of up to eight receptors and sometimes in close vicinity to dorsal eyes. Each receptor composed of a cell with a thick layer of microvilli and large nucleus. No retina or cornea observed. Photoreceptors separated by connective tissues and probably muscle cells (Figure 3D).

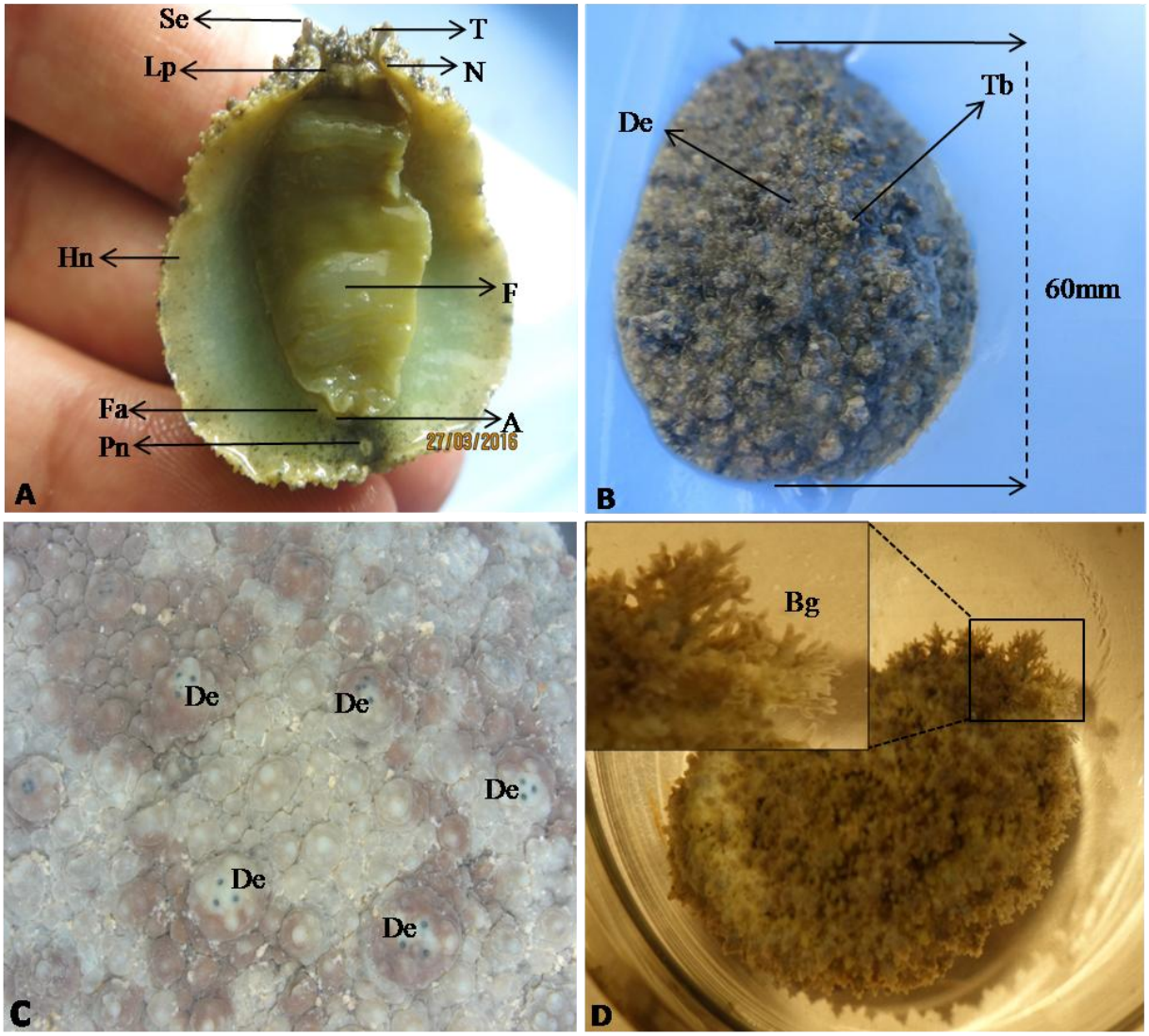

FIGURE 2. *Peronia persiae* **sp. nov**. External characters. **A-B.** Ventral and dorsal view of *Peronia persiae* **sp. nov. C.** Tubercles with dorsal eyes. **D.** Branchial gill at the rear of the back. Abbreviations: A = anus; Bg = branchial gill; De = dorsal eye; F= foot; Fa = female genital aperture; Hn = hyponotum; Lp = labial palp; N = notum; Pn = pulmonary aperture; Se = stalk eye; T = tentacle bearing eye; Tb = dorsal tubercle.

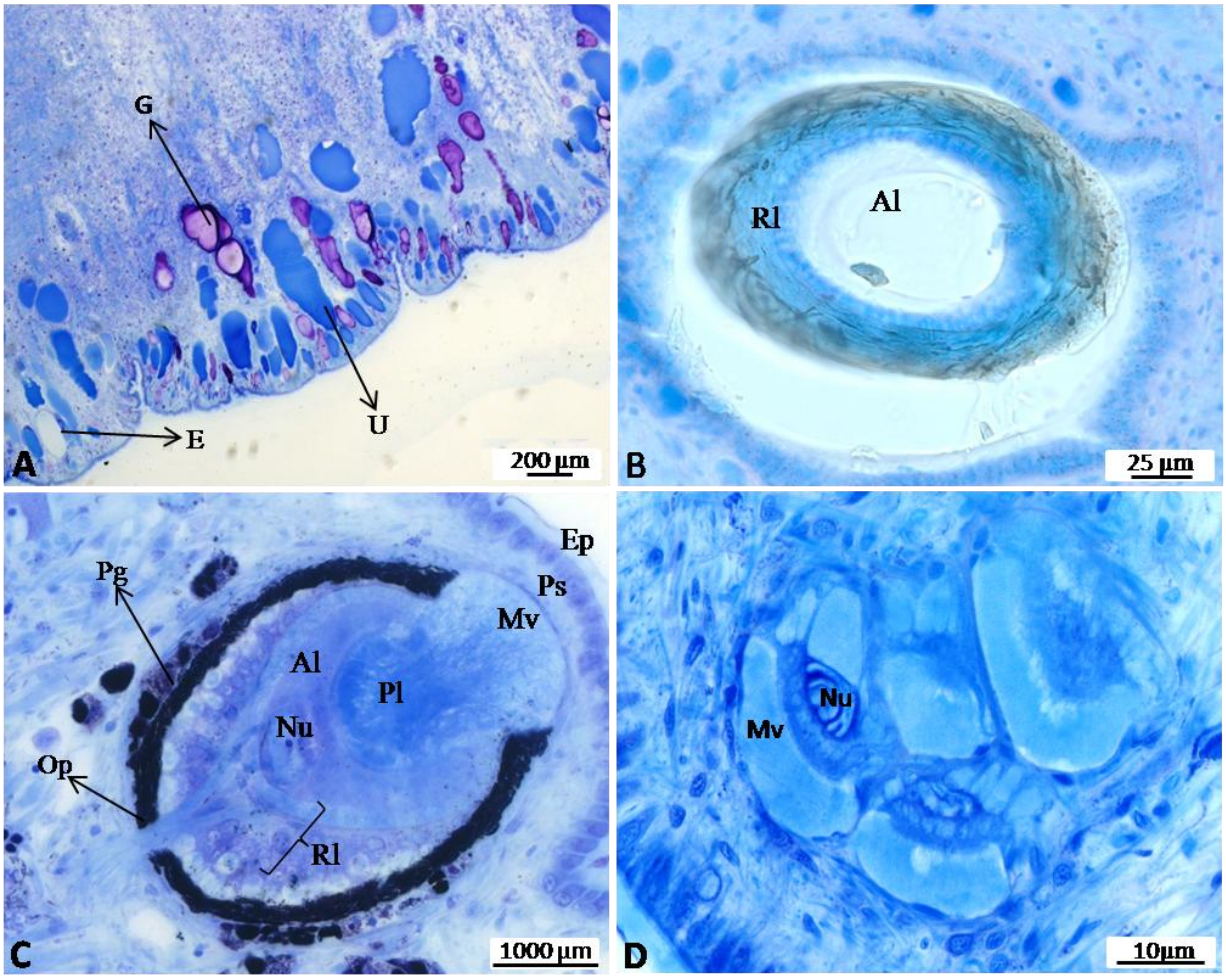

FIGURE 3. Histological sections of epidermis and sensory organs of *Peronia persiae* **sp. nov.**. A. Cross section of the mantle. B. Cross section of stalk eye. C. Longitudinal section of dorsal eye. D. Cross section of four dermal photoreceptors. Abbreviations: Al = accessory lens; E = empty unicellular gland; EP = epidermis; G = glandular vesicle consist of mucopolysaccharides (stained violet); U = unicellular gland; Mv = microvillus; Nu = nuclei; Op = optic nerve; Pg = pigmented layer; Pl = principal lens; Ps = pseudo cornea; Rl = retina layer.

Digestive system. Radula broad, with teeth lying on a thick cuticular membrane (Figure 4A). Interior of odontophore filled with red staining substance reminding of hyaline material or connective tissue ("elastic non-cellular substance", Awati & Karandikar 1948, p. 14). Odontophore flanked by the lateral cartilage-like radula cushions as well as pharyngeal muscles; radula cushions connected by a rigid area probably consisting of red stained connective tissue (Figure 4A). Radular formulae counted for 11 specimens; formulae ranging from 49 × 47.1.47

(specimen length alive 22mm) up to 71 × 87.1.87 (specimen length alive 65 mm). Rachidian teeth tricuspid with a main middle cusp and one distinctly narrow and long lateral cusp on both sides of main cusp (Figure 5A). Height of rachidian teeth about 50 µm. First inner lateral teeth smaller than other laterals (Figure 5B); height of unicuspid lateral teeth gradually increasing from about 60 µm to 75 µm and decreasing again towards the lateral rim. Lateral teeth with a conical shape seen from lateral view and inner side curved in a concave way (Figure 5C). Tips (seen from above) usually broadened and with a blunt, spatulate apex. (Figure 5A–B).

Salivary glands on both sides of the oesophagus with small ducts leading into pharynx close to the transition into the oesophagus; composed of many finger-like tubes combining into clusters and forming a grape-like structure. Glandular cells with reddish to violet staining granular contents (Figure 4B). Oesophagus with highly folded epithelium composed of ciliated columnar cells, covered in many areas with a thin homogenously staining (probably cuticular) layer - sometimes more greenish, sometimes bluish (Figure 4C). Oesophageal folds underlain with red stained connective tissue and surrounded first by a longitudinal and then by a circular muscular layer. Oesophagus entering the first part of the stomach. Stomach consisting of four parts. First part characterized by a thin epithelium; receiving the ducts of the dorsal and left lateral lobes of the digestive gland. Second chamber strongly muscular (Figure 4D), swollen and stratified; receiving the duct of the posterior lobe of the digestive gland. Third chamber funnel-shaped; pigmented on the outer side; its epithelium forming a highly folded structure with dendritic branches filling nearly completely the internal lumen (Figure 4E). The last chamber representing a small widened section at the beginning of intestine with only thin ridges internally and ciliated cells in the epithelium. Intestine forming two distinct long loops lying close together (Figure 6A–C), thus fitting best type II according to the definition of Labbé (1934). Epithelium with light blue stained cells and with violet stained goblet cells (Figure 4F). Digestive gland composed of many lobes; epithelium exhibiting cells which excrete substances stained in various shades of violet to blue (Figure 4G).

Reproductive system. Hermaphrodite gland (gonad) compact, whitish with bright dots representing the oogonia; located slightly on the left side of the visceral cavity, next to the diaphragm. Hermaphroditic duct originating from gonad and continuing to a widened area, possibly the ampulla, containing sperm, as well as a few oocytes; duct with small epithelial cells (Figure 7A, Figure 9A). Vas deferens forming a long tube starting from hermaphroditic duct in

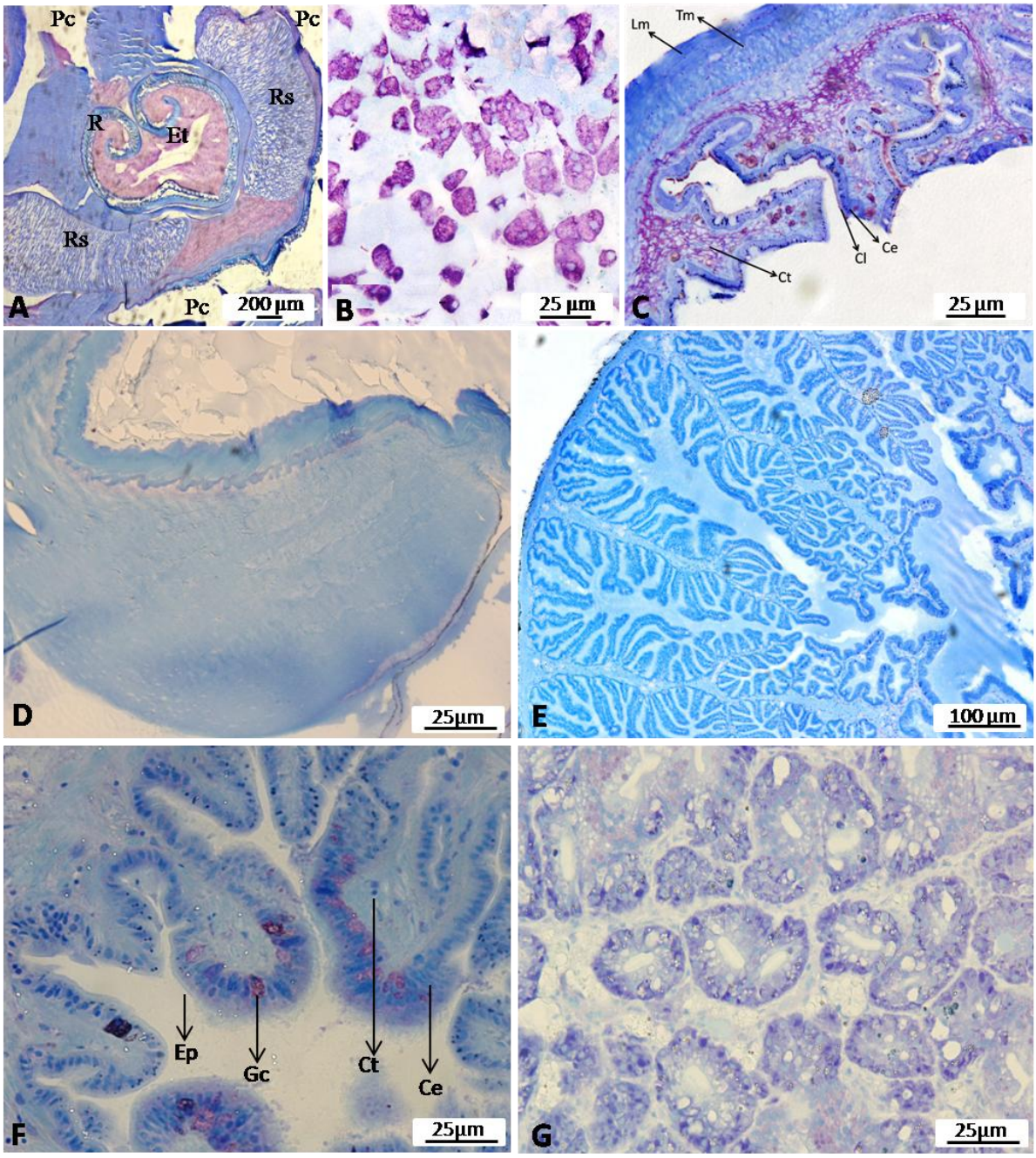

FIGURE 4. Histological sections of the digestive tract of *Peronia persiae* **sp. nov**. **A.** Cross section through the posterior region of the pharyngeal cavity and radula. **B.** Salivary gland cells. **C.** Cross section of oesophagus. **D.** Second chamber of stomach. **E.** Third chamber of stomach with highly folded (branched) epithelium. **F.** Cross section of intestine. **G.** Cross section of digestive gland. Abbreviations: Ce = columnar epithelium; Cl = cuticular lining; Ct = connective tissue; Ep = epithelium; Et = non-cellular elastic tissue; Gc = goblet cells; Pc =

pharyngeal cavity; R = radula bearing teeth; Rs = radula support; Tm = transverse muscle; Lm = longitudinal muscle.

the posterior third of body, running to the right side of the head, and ending in the penis; surrounded with a thick muscle layer and lined internally by ciliated cells. Prostate gland not observed. The anterior organs of male system consisting of the penial structure (penis with vas deferens and attached retractor muscle ending in penial sac) and penial accessory gland (glandular duct, hollow needle and end sac of penial gland).

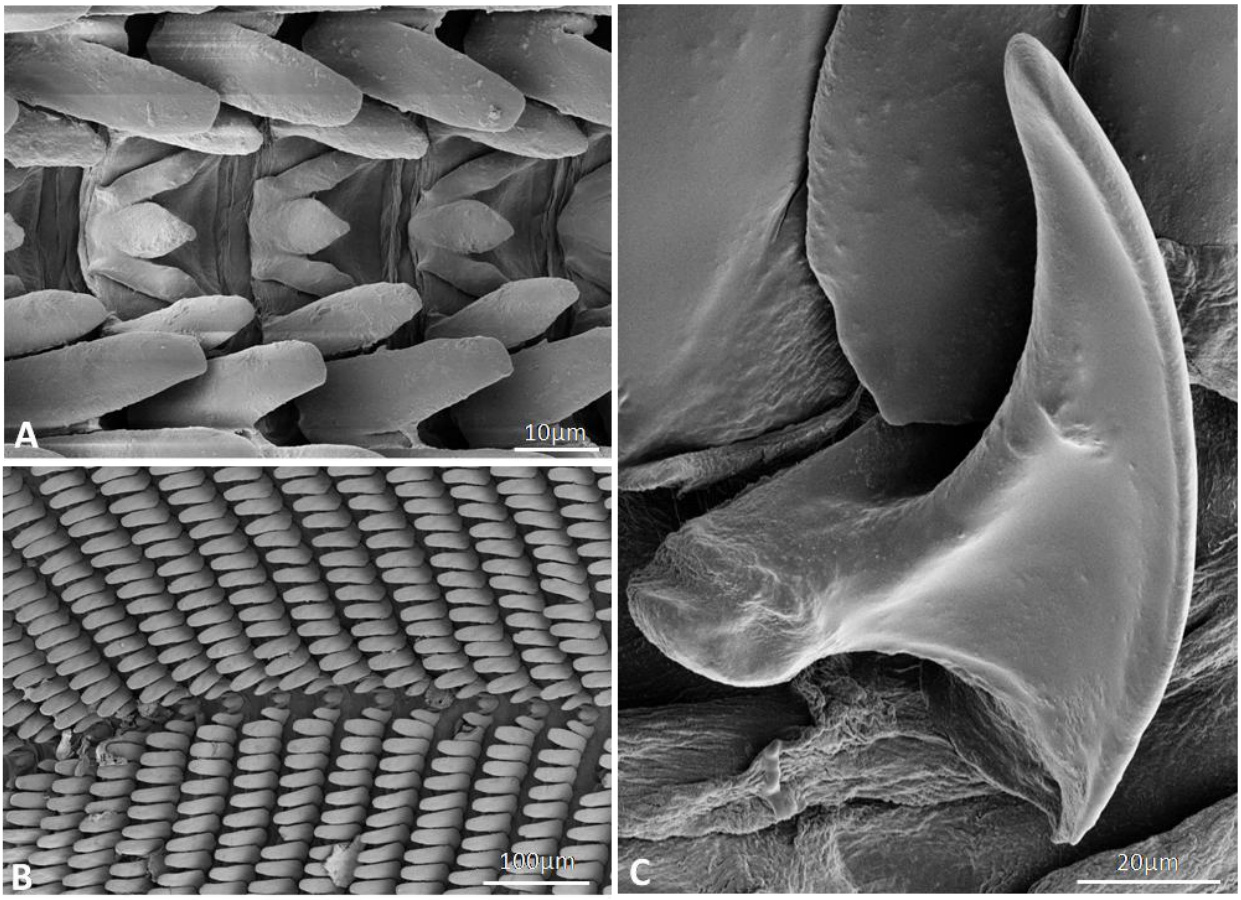

FIGURE 5. Scanning electron micrographs of the radula of *Peronia persiae* **sp. nov**: **A.** Rhachidian teeth. **B.** Lateral teeth. **C.** Lateral tooth.

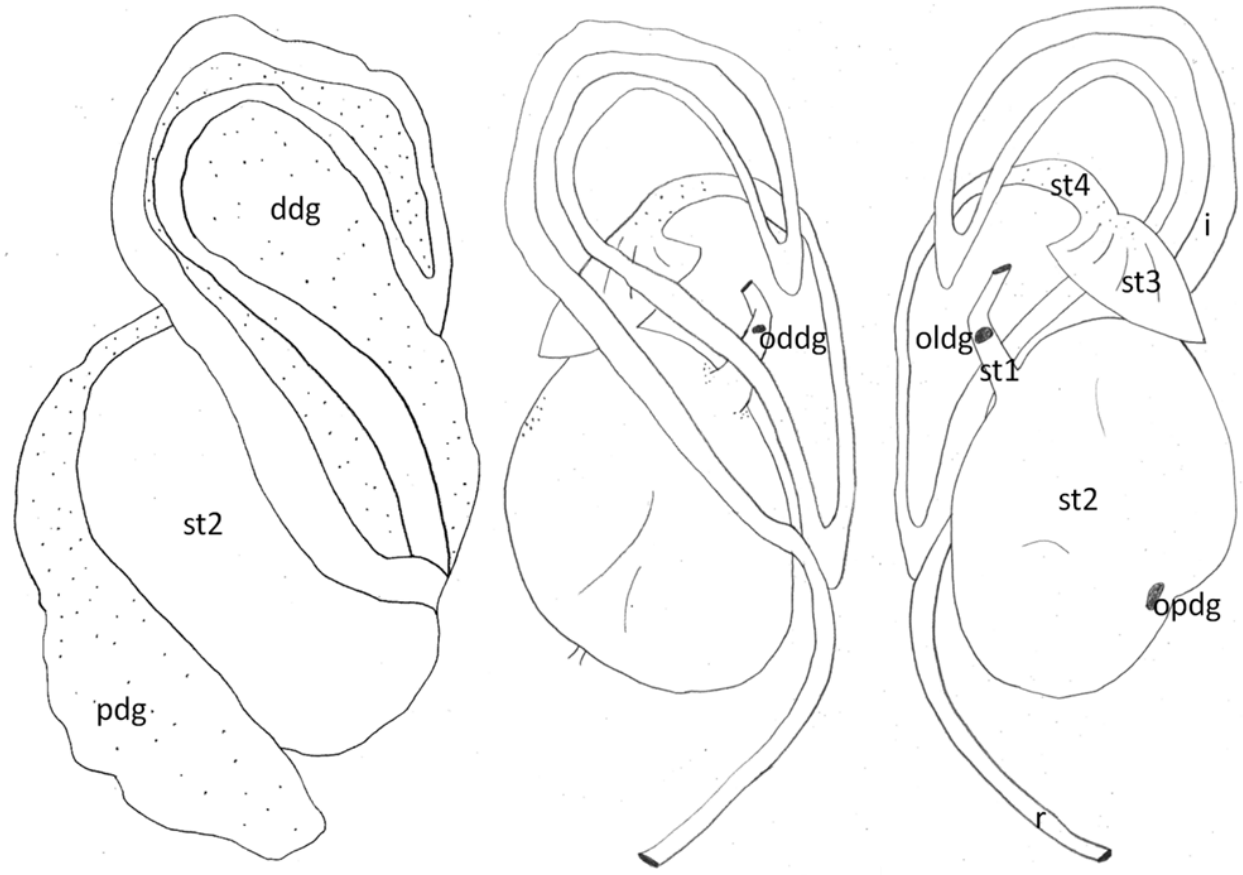

FIGURE 6. Digestive system of *Peronia persiae* **sp. nov.**. **A.** Dorsal view. **B.** Dorsal view with digestive gland removed. **C.** Ventral view. Abbreviations: ddg = dorsal lobe of digestive gland; i = intestine; oddg = opening of dorsal lobe of digestive gland; oldg = opening of lateral lobe of digestive gland; opdg = opening of posterior lobe of digestive gland; pdg = posterior lobe of digestive gland; r = rectum; st1 = stomach chamber 1; st2 = stomach chamber 2; st3 = stomach chamber 3; st4 = stomach chamber 4.

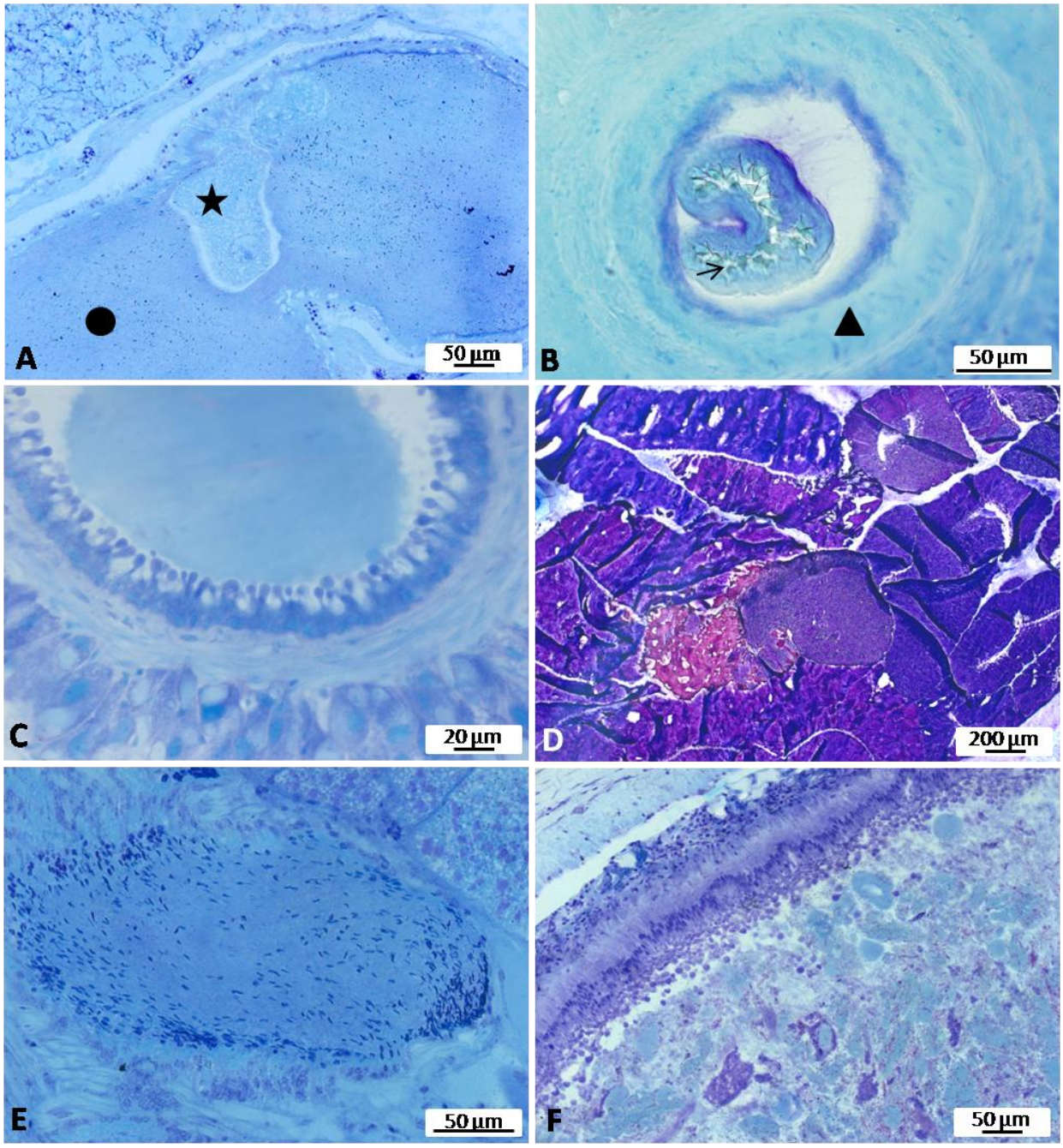

FIGURE 7. Histological sections of the genital system of *Peronia persiae* **sp. nov.**. **A.** Ampulla. **B.** Cross section of penis. **C.** Penial accessory gland duct. **D.** Female gland mass (mucus gland and albumen gland). **E**. Receptaculum seminis. **F**. Spermatheca. Symbols: arrow = penial hook; asterisk = eggs; dot = sperms; head arrow = muscular layer.

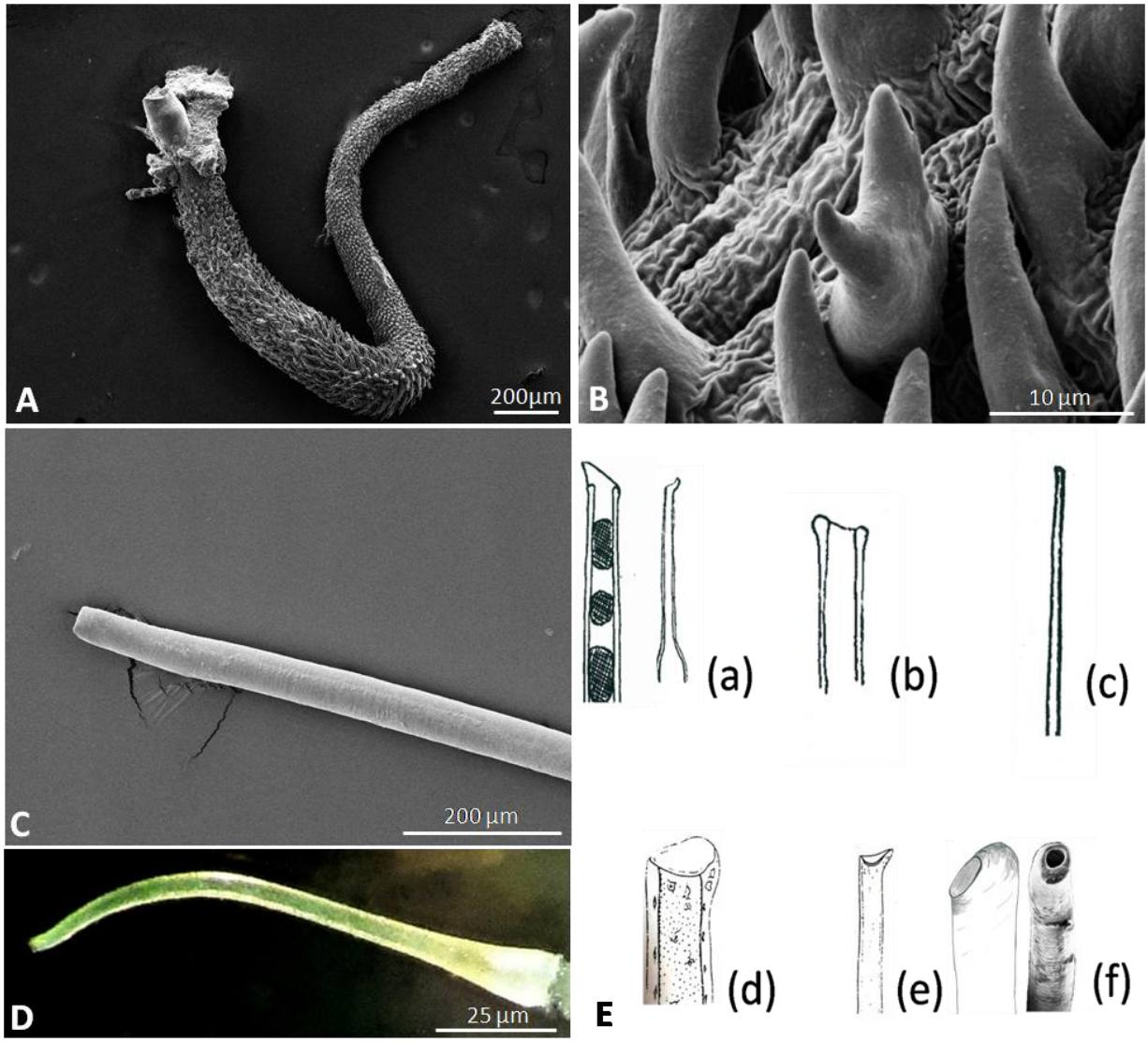

FIGURE 8. Cuticularized structures in the genital system of *Peronia persiae* **sp. nov.**. **A-C.** Scanning electron microscopy. **A.** Partly everted penis. **B.** Forked penial hook. **C.** Typical needle in penial structure. **D.** Light microscopy. Curved needle, observed only once. **E.** Shape of needle aperture retrieved from literature and current study: (a) *P. gondwanae* (Labbé 1934); (b) *P. indica* (Labbé 1934); (c) *P. verruculata* (Awati & Karandikar 1948); (d) *P. verruculata* (Plate 1893); (e) *P. branchifera* (Plate 1893); *P. persiae* **sp. nov**. (drawing and SEM picture).

Both complexes opening into a common vestibule and sharing same male opening (Figure 9B). Penis usually inverted into the vas deferens (Figure 7B) ending in the penial sac. The outer epidermis of penis covered with spines. During evagination, the usually outer layer turns to the outer side (Figure 8A). The hooks at the apical and distal areas are short and conical while the

rest of spines in the middle part are long, sharp and curved downward at the tips. Fork-shaped spines existing in different areas of the penis (Figure 8B).

Retractor muscle attached to the base of the penis in front part, running far into the posterior part of body. Penial accessory gland duct long, heavily coiled and swollen in posterior part, close to the opening; epithelium composed of columnar cells with apocrine secretion (Figure 7C). Light yellow needle apparatus straight, narrow and hollow; in one case tip slightly curved; length of needles around 1.3 mm (Figure 8C–D). The opening of needle usually blunt, only in one individual of sickle shape (Figure 8E). Sac-like structure without papillae.

Proximal spermoviduct folded and embedded within the female gland mass. The latter composed of capsule gland (albumen gland), followed by membrane gland, leading into mucus glands (Figure 7D, Figure 9A). Membrane gland located ventrally underneath capsule gland, composed by columnar cells filled with reddish granules, mucus gland composed of columnar cells containing basal nucleus and many small, ovoid, violet staining granules. Presence of spiral glands could not be verified.

Whitish receptaculum seminis composed of small epithelial cells, with attached sperm heads (allosperms) (Figure 7E). Brownish round to elongate spermatheca (or bursa copulatrix in the sense of Schmekel 1971) connecting to short distal oviduct; greenish staining contents present inside spermatheca; epithelium composed of apocrine secreting cells (Figure 7F). Female opening near the anus slightly to the right side.

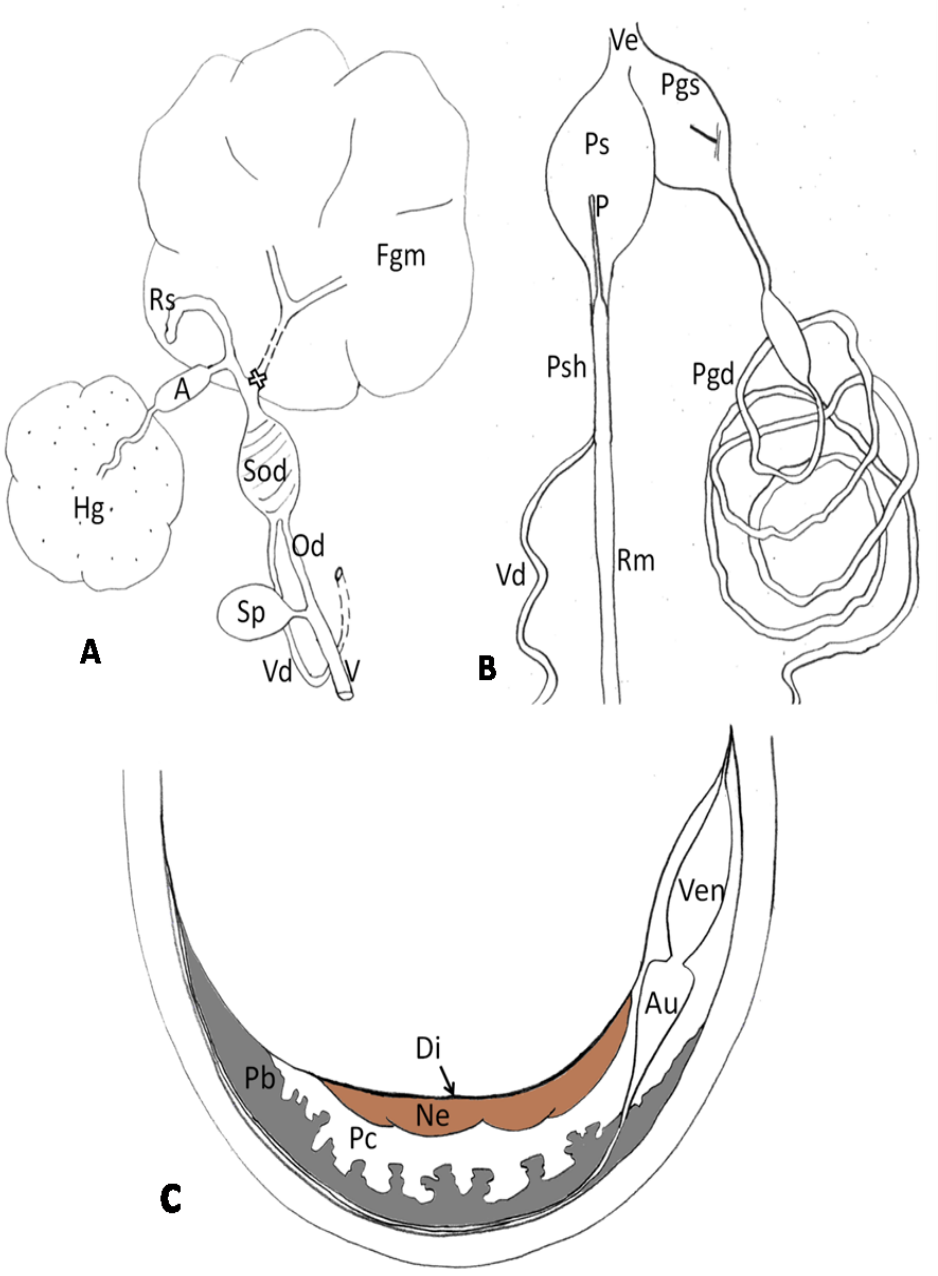

FIGURE 9. Reproductive and excretory system of *Peronia persiae* **sp. nov. A-B**. Female part of the hermaphroditic genital system. **B.** Male copulatory parts. **C.** Posterior part of the body opened and displayed from dorsal side. Abbreviations: A = ampulla; Au = auricle; Di = diaphragm; Fgm = female gland mass; Hg = hermaphrodite gland; Ne = nephridium; Od = oviduct; P = penis; Pb = pulmonary cavity branches; Pgd = penial gland duct; Pgs = penial gland sac containing needle; Ps = penial sac; Psh = penial sheath; Rm = retractor muscle; Rs = receptaculum seminis; Sod = spermoviduct; Sp = spermatheca; V = vagina; Vd = vas deferens (towards head); Ve = vestibule; Ven = ventricle. Connection from spermoviduct to female gland mass not clearly seen and indicated here with dotted line.

Circulatory and excretory system. Heart on the right side, divided into an anterior ventricle and posterior auricle within pericardial cavity. Nephridium posterior to the diaphragm. Extension of nephridium larger on the right side, than on the left side (Figure 9C).

Phylogeny. The phylogenetic analyses of the concatenated dataset (Figure 10) covering 11 of the 16 acknowledged genera and including 457 sequences, provide evidence of the monophyly of *Peronia* with high bootstrap support in the ML analyses (99) and close relationship with a clade formed by the monophyletic *Wallaconchis* (96), *Alionchis* (100) and *Paromoionchis* Dayrat & Goulding, 2019 (100). The close relationship of the four genera is supported by 97 BV. One specimen retrieved from NCBI as *Scaphis* sp. groups within the genus *Peronia*. Further monophyletic genera are *Peronina* (100), *Melayonchis* (76), *Marmaronchis* (100), *Onchidina* (100), and *Onchidella* (99). Monophyly of *Onchidium*, as shown in Dayrat *et al.* 2016 with a reduced dataset of Onchidiidae, cannot be seen in our tree, however misidentification of the paraphyletic genus *Platevindex* H. B. Baker, 1938 with members grouping with *Onchidium* cannot be excluded. In this overall analysis, *P. persiae* **sp. nov.** is clearly separate from all other *Peronia* species (Figure 10). These results are confirmed by analysing the reduced datasets including only *Peronia* sequences and running the analyses with COI only (Figure 11, Table 3), or the concatenated alignment with COI and 16S, and analyses of distance values (Figure 12). In these two analyses, the 11 specimens of the new species always group as a separate clade, usually as sister of *Peronia* sp. 3, and close to *Peronia* sp. from Singapore (COI, Figure 11) or close to *Peronia* sp. 4 and sp. 5 from Mozambique (concatenated data set, Figure 12).

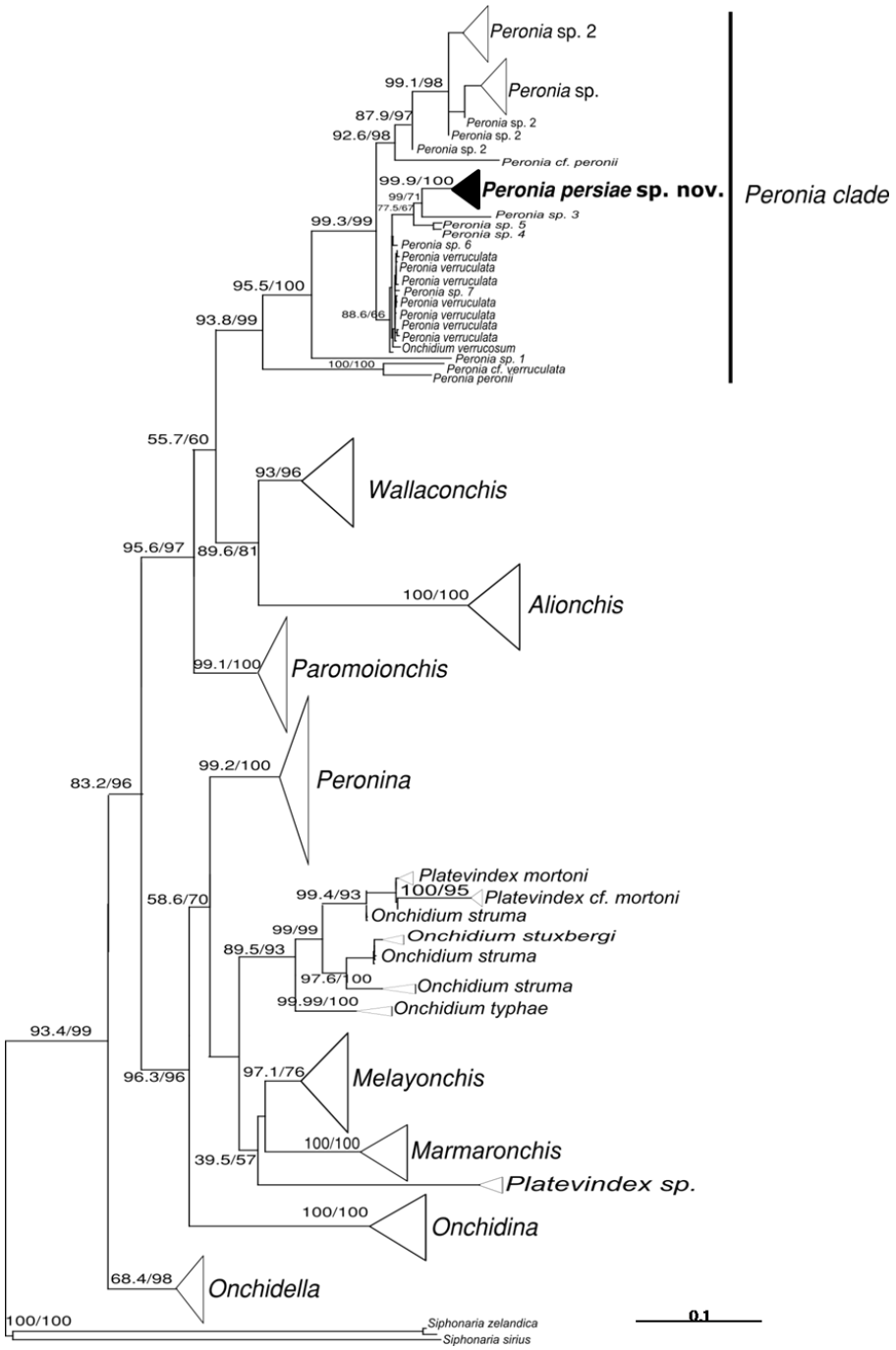

FIGURE 10. Phylogenetic reconstruction of Onchidiidae resulted from Maximum likelihood (ML) analysis of concatenated data set (COI and 16S) with *Siphonaria* as outgroup. *Peronia persiae* sp. highlighted in bold. Numbers before and after slash indicate approximate likelihood ratio test (SH-aLRT) and ultrafast bootstrap values respectively.

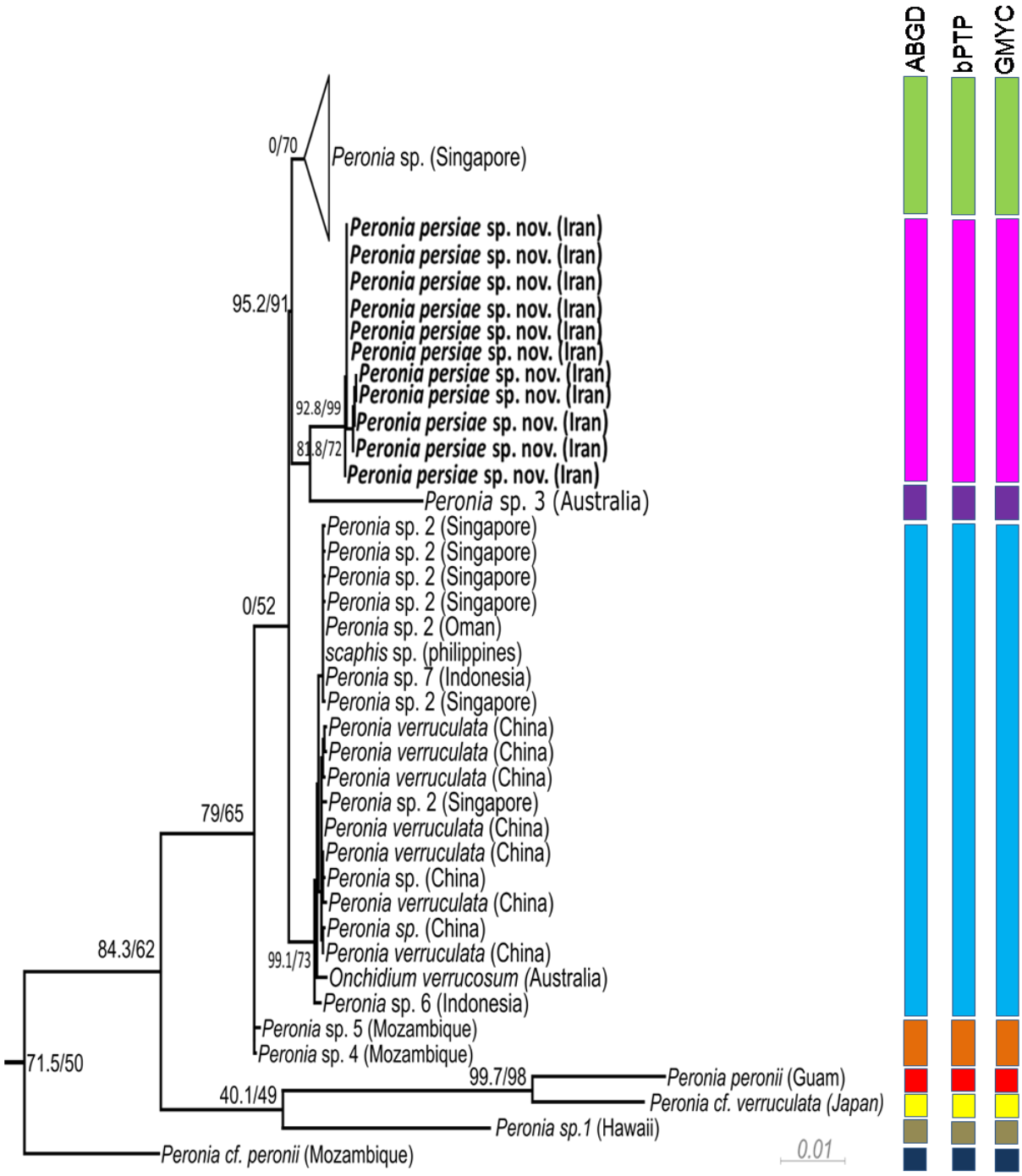

FIGURE 11. Maximum likelihood tree of the genus *Peronia* based on COI data set, *Wallaconchis graniferus* used as outgroup (not shown). Groups resulting from ABGD, GMYC, and bPTP tests indicated on the right side. Numbers before and after slash indicate approximate likelihood ratio test (SH-aLRT) and ultrafast bootstrap values respectively.

Species delimitation. All three species delimitation methods applied to the COI (Figure 11) and 16S datasets indicate nine well supported groups in the genus *Peronia* (Figures 11–12, S1–S4). The Iranian specimens always form a separate taxon from *P. verruculata* specimens or any other *Peronia* species. The result of the ABGD test based on COI data shows a single and obvious barcode gap: intraspecific variability is less than 2% and interspecific variability more than 4%. The distance between the new Iranian and other close related species, *Peronia* sp. 4 and *Peronia* sp. 5, ranges between 7.0-8.0% (Table 3). All three tests applied on the separate two genes (Figure 12) show the same results: one Singapore group, assigned to *Peronia* sp., is indicated as a separate species. Another group, assigned to *Peronia* sp. 2 from Singapore and Oman, together with sequences assigned to *P. verruculata* and *Onchidium verrucosum* (misspelling of *P. verruculata*) from China and Australia, and a few sequences from different localities (*Peronia* sp. from China, *Peronia* sp. 6 and *Peronia* sp. 7 from Indonesia, *Scaphis* sp. from Philippine Islands) are considered to be a single species. Results of GMYC and bPTP analysis, using the 16S data set, shows *Peronia* sp. 7 from Indonesia and *Scaphis* sp. as a separate group. *Peronia* sp. 4 and sp. 5 from Mozambique are indicated also as a single and distinct species; however, this last result was not retrieved in the GMYC and bPTP analysis using the 16S data set (Figure 12). *Peronia* sp. 3 from Australia and *Peronia* sp. 1 from Hawaii also are distinct species in all analyses. *Peronia* cf. *peronii* from Mozambique is considered as a distinct species, when applying only the CO1 dataset. This result is not confirmed in the GMYC and bPTP analysis using only the 16S data set. *P. peronii* from Guam (indicated as a separate species) clusters with a sequence of *Peronia* cf. *verruculata* from Japan (*Peronia* sp. according to Dayrat *et al.* 2016), which also forms a separate species (confirmed in all analyses).

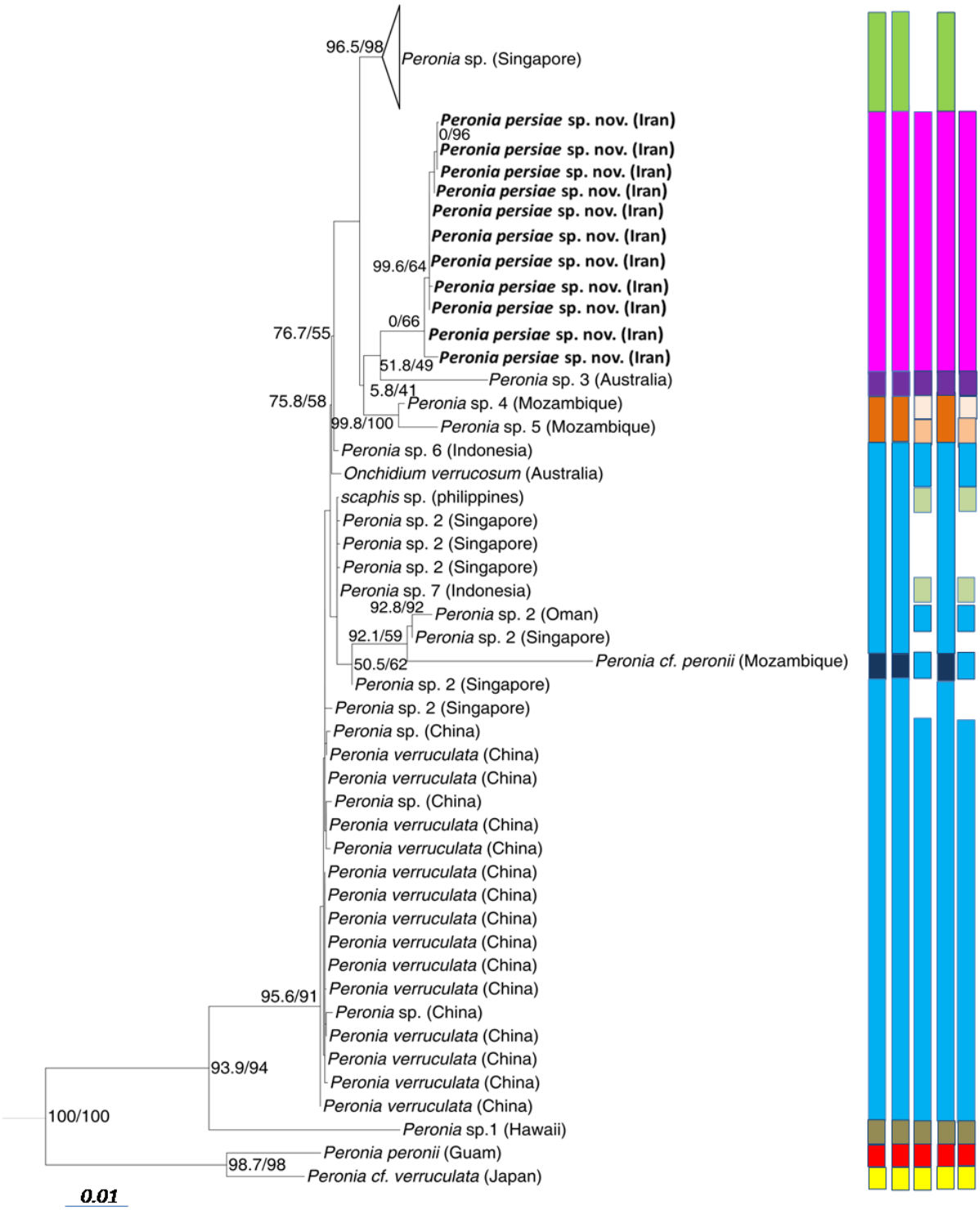

FIGURE 12. Maximum likelihood tree of the genus *Peronia* based on the concatenated alignment (COI and 16S). Groups resulting from ABGD, GMYC, and bPTP tests indicated on the right side. Numbers before and after slash indicate approximate likelihood ratio test (SH-aLRT) and ultrafast bootstrap values respectively.

TABLE 3. Intra- and interspecific pairwise genetic distances. Ranges from minimum to maximum distances are indicated (as percentages). * indicating a group of sequences comprising *Peronia verruculata* (China), *Onchidium verrucosum* (Australia), *Peronia* sp.2 (Oman), *P*. sp.6 (Indonesia), *P*. sp.7 (Indonesia), *P*. sp (China), *Scaphis* sp. (Philippine Islands), and *Peronia* sp.2 (Singapore).

	Peronia sp. Clade (Singapore)	*Peronia verruculata* group*	*Peronia* sp. 4, sp. 5 (Mozambique)	*Peronia persiae* **sp. nov.** (Iran)	*Peronia* sp.3 (Australia)	*Peronia* cf. *peronii* (Mozambique)	*Peronia* sp.1 (Hawaii)	*Peronia peronii* (Guam)	*Peronia* cf. *verruculata* (Japan)
Peronia sp. Clade (Singapore)	0-9								
Peronia verruculata group*	4-6	0-2							
Peronia sp. 4, sp. 5 (Mozambique)	5-7	6-9	0-1						
Peronia persiae **sp. nov.** (Iran)	6-9	8-12	7-8	0-1					
Peronia sp.3 (Australia)	10-11	10-12	10-11	10-11	0				
Peronia cf. *peronii* (Mozambique)	15	15-17	14-15	14-15	16.40	0			
Peronia sp.1 (Hawaii)	17-18	15-18	18-19	17	16.70	20.90	0		
Peronia peronii (Guam)	19-20	20-22	19	21	21.20	18.90	21.40	0	
Peronia cf. *verruculata* (Japan)	20	20-21	19	20	21.00	19.31	18.90	13.50	0

Discussion

Morphology. Although several species of *Peronia* have been described, most information is available only for *P. verruculata* and *P. peronii*. Nevertheless, *P. persiae* **sp. nov.** can be distinguished from all described species by several features (see Table S1) and resembles most *P. verruculata*. Our new species has branchial gills covering only the posterior part of the body (Figure 2B) as is described for many *Peronia* species (Farran 1905; Britton 1984). Only *P. peronii* has gills scattered all over the notum (Plate 1893; Labbé 1934; Solem 1959). Similar to *P. verruculata, P. persiae* **sp. nov.** lacks spicules in the notum (Awati & Karandikar 1948). Information about this character is missing for *Peronia anomala* Labbé, 1934, *Peronia lata* Labbé, 1934 and *Peronia branchifera* Plate, 1893; however, all other described *Peronia* species possess spicules. The visceral cavity in *P. persiae* **sp. nov.** is strongly pigmented like in *P. verruculata* (Awati & Karandikar 1948), while the visceral cavity of *P. peronii* is not pigmented (Labbé 1934; Plate 1893). Various photoreceptive systems are thoroughly described for *P. verruculata* (Katagiri *et al.* 1985) including stalk eyes, dorsal eyes, free dermal photoreceptor cells in the notum, and photosensitive neurons in the central nervous system. At least the first two types of photoreceptors are present in all *Peronia* species (Table S1). One dorsal tubercle has several eyes and these tubercles are scattered over the dorsal notum. Only *P. lata* has one dorsal eye per tubercle and thus differs from our new species.

P. persiae **sp. nov.** lacks the pharyngeal wall teeth, which are present in *P. verruculata* (Awati & Karandikar 1948). Additionally, the new species has a larger radula with more rows and more teeth per row, when comparing *P. verruculata* specimens of same size with our specimens (both 65 mm): 71×87.1.87 versus 65×66.1.66 (Awati & Karandikar 1948). The lateral cusps on both sides of the rachidian teeth are more elongate and pointed, and the main cusp is much more developed in *P. persiae* **sp. nov.** than in *P. verruculata* (Plate 1893; Awati & Karandikar 1948; Britton 1983), thus similar to *P. peronii* (Labbé 1934). The concave internal part of the lateral teeth was not mentioned before (Figure 5C) and might be a special character of the new species. Most onchidiid species have a stomach divided into four parts (Weiss & Wägele 1998; Dayrat *et al.* 2016). However, stomachs with three or two parts are also reported (Dayrat 2010a; Dayrat 2010b). Awati & Karandikar (1948) considered the third and fourth parts as one single chamber in *P. verruculata*. The stomach of the here investigated *P. persiae* **sp. nov.** has four parts in which the fourth has a ciliated epithelium. The intestine with two loops can be assigned to type II.

Descriptions for *P. peronii* and *P. verruculata* vary between the more common intestinal type I (with one loop) and type II (Plate 1893; Labbé 1934; Marcus & Marcus 1959).

The position, symmetric or asymmetric, of the nephridium is mentioned as a distinguishing feature for *Peronia* species. *P. persiae* **sp. nov.** has an asymmetric nephridium with the right side larger than the left side. According to the illustration of *P. verruculata* (Awati & Karandikar 1948, Figure 38) the nephridium is larger on the left side. However, Labbé (1934) mentioned a symmetric nephridium for *P. verruculata*, as well as for *P. peronii.* These differences in the description show that the nephridium can vary and therefore cannot be considered as a diagnostic character.

In many heterobranch species, a part of the hermaphroditic duct is swollen into an ampulla, in which autosperm is stored before release during copulation. An ampulla is not described for any Onchidiidae species except a specimen investigated by Marcus (1971) and assigned to *Onchidium simrothi* Plate, 1893 (Marcus 1971). This specimen was recently assigned to the new genus *Wallaconchis*: Goulding *et al.* (2018b) re-examined the type material of *O. simrothi* and subsequently considered this name as a *nomen dubium*. They did not mention an ampulla for any *Wallaconchis* species. It seems that this character is difficult to find in onchidiids, or it is only present in a few species. The feature that we describe here in the gonoduct, filled with irregularly lying sperm, is very similar to the structure that is described by Schmekel (1971) as ampulla in Nudibranchia. We herein report for the first time the existence of ampulla in *Peronia*.

The vas deferens often exhibits an enlarged special area (considered as prostate) with glandular cells producing prostatic material (e.g., Wägele & Willan 2000). In histological investigations, prostatic cells are visible by their typical glandular cells. Interestingly, no prostate was detected in *P. persiae* **sp. nov.**, while Awati & Karandikar (1948) described it in detail for *P. verruculata*. Unfortunately, no further information about the prostate is available for any other *Peronia* species. The penial complex usually has the same structures in most Onchidiidae species, including members of *Peronia*. The epidermis of the penis is usually covered by spines. We observed some differences in *P. persiae* **sp. nov.** such as the penial hooks in the anterior and posterior part of the penis being larger than those of the middle region. Short spines in the posterior part are not described in *P. verruculata*. Additionally, some hooks are fork-shaped, and these special forms are scattered all around the penis. The fork-shaped hooks are not known for any other *Peronia* or onchidiid species. Another distinguishable character in *P. persiae* **sp. nov.**

is the absence of calcareous granules which were described on the hooks of the anterior part of the penis in *P. verruculata* (Awati and Karandikar 1948; Britton 1984).

The penial gland duct of a member of Onchidiidae is investigated histologically here for the first time. Its function might be provision of substances to help in sperm transfer. No papillae of the penial gland have been observed in *P. persiae* **sp. nov.**, while this structure is described for *P. verruculata* (Britton 1984) and *Peronia gaimardi* Labbé, 1934 (Labbé 1934). *Peronia ferruginea* Lesson, 1831 completely lacks the penial gland (Lesson 1830, Britton 1984). The colour of the penial needle in *P. persiae* **sp. nov.** is light yellow, like in *P. verruculata* (Awati & Karandikar 1948), but dark brown in *P. peronii* (Plate 1984). The shape of the penial needle opening can vary among the *Peronia* species (Figure 8E). It is shovel-shaped in *P. branchifera*, oblique or shovel shaped in *Peronia gondwana* Labbé, 1934, oblique in *P. indica*, and unilateral thickened in *P. verruculata*. *P. persiae* **sp. nov.** has two new different shapes of needle openings, which are not described from any other *Peronia* species. In most individuals of *P. persiae* **sp. nov.** it is blunt and only in one individual it is sickle shaped. All other parts of the genital systems are similar to the ones described for *P. verruculata* and *P. peronii*.

Based on these combinatory anatomical differences (Table S1), we cannot assign our specimens to any described *Peronia* species and therefore consider it as a distinct new species.

Molecular data. This first molecular analysis of the Onchidiidae including all available sequences and covering 11 genera, provide good evidence for the monophyly of *Peronia* and nearly all other onchidiid genera. *Platevindex* and *Onchidium* are not monophyletic, thus supporting former analyses, based on smaller data sets without the five recently described genera *Wallaconchis*, *Alionchis*, *Paromoionchis*, *Melayonchid* and *Marmaronchis* (Dayrat *et al*. 2017, 2018, 2019; Goulding *et al*. 2018a; Goulding *et al*. 2018b). Species delimitation tests clearly show that *P. persiae* **sp. nov.** is a distinct species. It does not group with any of the other specimens assigned to *P. verruculata* or other, unidentified *Peronia* sequences. It thus supports the results based on the morphological analysis. Additionally, our results indicate the presence of several further unidentified and potentially undescribed species of *Peronia*. Different species delimitation analyses (GMYC, ABGD, bPTP) based on COI and 16S genes, detected nine species within this genus. It is not clear whether these groups represent already described species, because no sequences are available for many recognized *Peronia* species and therefore a revision of the genus is highly needed.

Conclusions

Integrative species delimitation analyses are well-established in heterobranchs (e.g. Jörger *et al.* 2012, Krug *et al.* 2013; Padula *et al.* 2014). Investigating morphology with different methodologies and combining these results with those obtained from an array of suitable molecular approaches can provide good evidence in species delimitation and descriptions. Our analysis has shown for the first time the presence of a new *Peronia* species in the intertidal flats along the southern coast of Iran. With this new species, ten *Peronia* species are considered as valid. Future analyses will show whether any of the sequences in our analysis confirmed here to belong to the genus *Peronia* can be assigned to one of the former described species. Whether *P. persiae* **sp. nov.** is endemic to Iran or has a wider geographic distribution is not known, more studies in the Persian Gulf and adjacent areas are necessary. The lack of records of other onchidiid species in this region suggests that *P. persiae* **sp. nov.** may have evolved certain adaptations to live in the extreme environmental conditions of the intertidal flats of Iran.

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Supplementary information:

TABLE S1. Characterized features of valid species within the genus *Peronia*. Abbreviation in brackets indicates authors and year of publication: A = Awati & Karandikar 1948, At = Attaran *et al*. 2015, B = Britton 1984, Bi = Bitaab *et al*. 2015, Br = Bretnall 1919, C= Cuvier 1804, F = Farran 1905, H = Hyman 1999, L = Labbé1934, Le = Lesson 1830, M = Marcus & Marcus 1959, Pa = Patil & Kulkarni 2013, P = Plate 1893, S = Solem 1959, W = White 1951.

Species	Original name	Maximum length of body (mm)	Dorsal eye
P. anomala	Peronia anomala Labbé, 1934	10 (L)	-
P. branchifera	Oncidium branchifer-um Plate, 1893	27.5 (P), 30 (L)	8 tubercles with eyes (P), 2-4 eyes per tubercle (L & P)
P. gaimardi	Peronia gaimardi Labbé, 1934	84 (L)	-
P. gondwanae	Paraperonia gondwanae Labbé, 1934	32 (L)	3-4 eyes per tubercle (L)
P. indica	Quoyella indica Labbé, 1934	22 (L)	3-4 eyes per tubercle (L)
P. lata	Scaphis lata Labbé, 1934	28 (L)	single eye at each tubercle (L)
P. ferruginea	Onchidium ferrugineum Lesson, 1831	30 (L), 38 (Le)	2-3 or 4 eyes per tubercle (L)
P. peronii	Onchidium peronii Cuvier, 1804	155 (M),122 (L),130 (C), 53 (W), 155 (M), 104 (Br), 139.7 (P), 100 (At), 20 (Pa), 85 (Bi)	4-5 eyes per tubercle (P)
P. verruculata	Onchidium verruculatum Cuvier, 1830	31 (Br), 40 (B), 50 (P), 69 (A), 70 (Pa)	6 tubercles with 2-5 eyes (F & L), yellow tipped papilla bearing 3-4 black eyes (H), 1-4 eyes per tubercle (Br), single or 1-6 eyes per tubercle (A), 6-7 eyes per tubercle (P)
Peronia persiae **sp. nov.**	-	65	maximum 16 tubercles, 2-4 eyes per tubercle

CColour of notum and hyponotum	Type of intestine	Integument
-	type II (L)	thin (L)
middle of notum yellow with black brown patches, outer part black brown (P), dirty yellow notum (L)	type I (P & L)	-
dirty yellow with black spots (L)	type I (L)	thick, large spicules widely spaced (L)
greyish yellow (L)	type V, sometimes type I (L)	thick, with large spicules (L)
-	type V (L)	with spicules (L)
uniform greenish yellow (L)	type I (L)	
notum light yellow (L), notum intense ferruginous red (Le), yellowish white with black ventral head (L & Le)	type I (L)	thin, with large spicules (L), very thick and fleshy (Le)
very variable: greyish or blackish yellow or marble with spots (L), greenish or blackish, hyponotum pale yellow (C), green grey with light patches (M), olive (Br), black to light brown (P), dark green to brown with grey patches (At)	type I or II (M), type I (L), type I (P)	with irregular spicules (L)
yellow greyish with yellow or brown spot, hyponotum yellowish white (L), olive with brown pattern, hyponotum light olive (Br), brown with uniform red-brown or reddish markings and yellow to brown notum margin, hyponotum bluish grey (H), grey to blue grey with stripes or black mottling (Br), grey yellow with black brown patches(P), brown (Pa)	type I or rarely type II (L), type II (H), type I, occasionally type II (Br), type I (P), type I (A)	thick, without spicules (A)
notum muddy green to grey, hyponotum yellow green	type II	thick, without spicules

Penial hooks of penis	Papillae at base of	Shape of aperture of needle and colour	Male pore	Branchial gill position and form
-	-	-	-	posterior 1/4 (L)
-	absent (P)	shovel-shaped (P)	one pore on left side of right tentacle (P)	posterior 1/6 (P), long finger shape extensions (L & P)
-	present (L)	-	one pore on left side of right tentacle (L)	-
-	-	unilateral thickening (L), needle narrow	-	Posterior (L)
-	-	oblique (L)	two separate pores (L)	posterior 1/6 (L)
-	-	-	-	Posterior (L)
-	present (L), absent (B)	-	-	posterior 1/6 (L & Le), short tubercles, gathered in small bundles of five to six (Le)
-	-	needle black brown (P)	-	all over the notum (L, P & S), spongy appearance (C), close to posterior mantel border (Br)
anterior part with hooks, posterior part without hooks (Br), anterior and posterior parts with hooks, but posterior with large spines and granules (A), without “chondroid” elements (P)	present (Br)	unilateral thickening (P), needle yellow and very narrow (A)	one pore on left side of right tentacle (A)	10 gills in posterior 1/4-1/5 (F & L), short gills at posterior side (Br), posterior 1/4, short and finger shape (P)
posterior spines much larger than anterior and both parts without calcareous granules	absent	Blunt and only one sickle shaped, needle light yellow	one on left side of right tentacle	15 branchial gills, posterior 1/6, very short, branched from the base

Rectal gland	Visceral cavity	Position of respiratory opening in posterior part	Receptaculum seminis (vesicule seminale)	Spermatheca (receptacle seminalis)	Prostate
-	slightly pigmented (L)	-	-	-	-
absent (P)	pigmented (L)	median (P), median (L)	Conical (P)	large (P)	-
	non or partially pigmented (L)	median (L)	-	-	-
-	-	-	-	-	-
-	light brown pigmented (L)	to the right side (L)	-	-	-
-	non pigmented (L)	to the right side (L)	-	-	-
present (B)	non pigmented or light brown or weakly black pigmented (L)	-.	-	-	-
-	non pigmented or scattered pigmentation (L & P)	median or a little bit to the right side (L), median or slightly to the right side (M), median (P)	large,12-15 mm (L), blind sac (M), large with thin stripe (P)	large,12-15 mm (L), much larger than receptaculum (M), larger than receptaculum(P)	-
absent (P)	strongly pigmented (L), pigmented from light to pronounced colour (Br)	median (L), median (H), median (P)	large, tubular structure, 10mm (L), small and smooth (H), large, tubular (P), finger shaped (A)	Large (H), short and stalked (P), round (A)	Unidentified structure (no name provided)
absent	strongly black pigmented	median	present	Roundish to elongate and short stalked	Absent

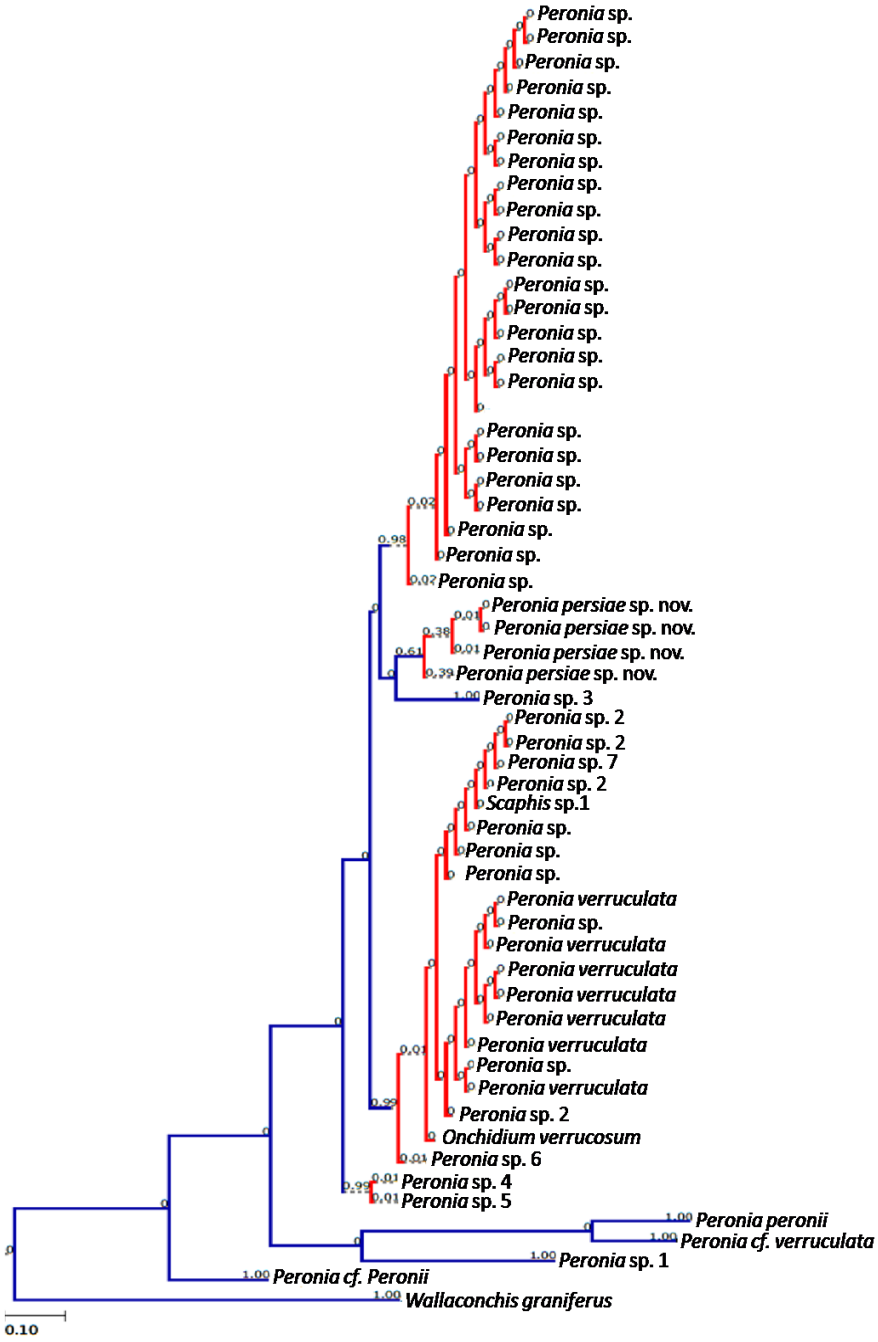

FIGURE S1. Rooted ultrametric tree from Bayesian analysis with branches coloured according to the bPTP species delimitation analysis for the genus *Peronia* based on COI alignment. Bayesian support values are shown above the branches. Blue lines indicate branching processes among species, red lines indicate branching processes within species. *Wallaconchis graniferus* as outgroup and identical sequences are removed.

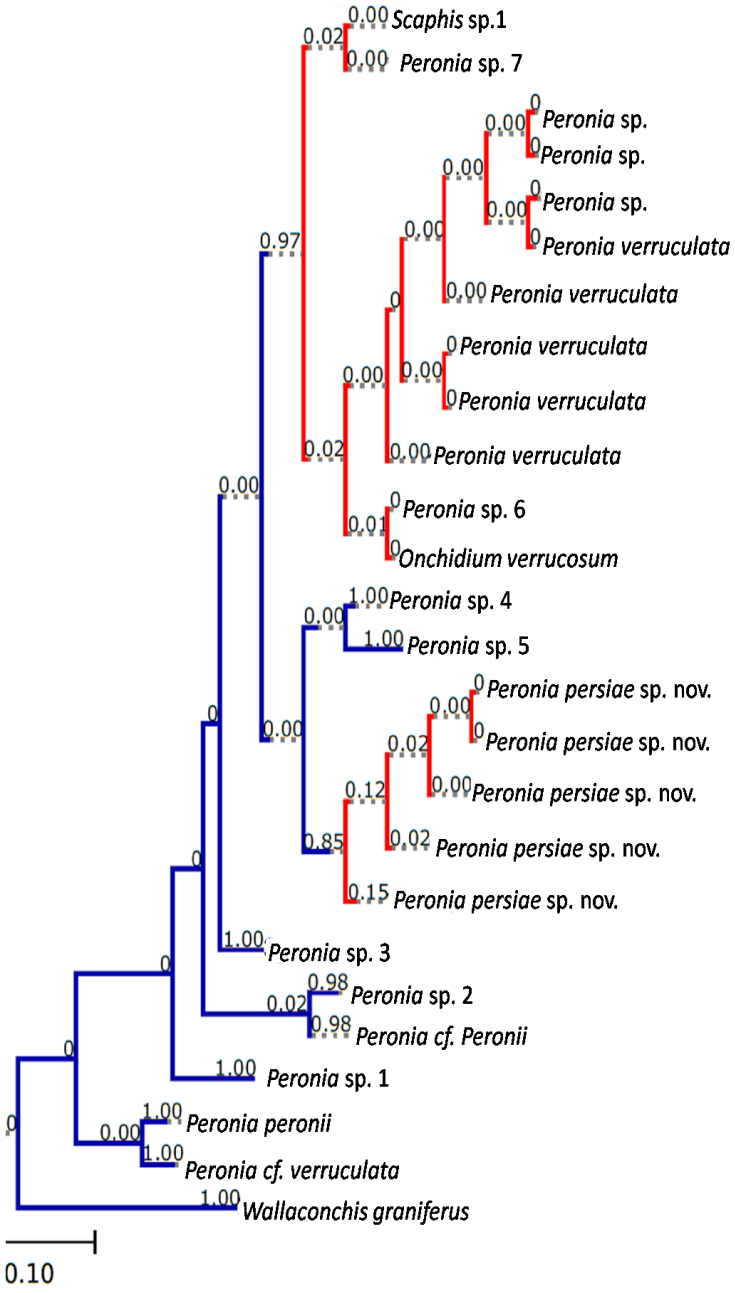

FIGURE S2. Rooted ultrametric tree from Bayesian analysis with branches coloured according to the bPTP species delimitation analysis for the genus *Peronia* based on the 16S alignment. Bayesian support values are shown above the branches. Blue lines indicate branching processes among species, red lines indicate branching processes within species. *Wallaconchis graniferus* as outgroup and identical sequences are removed.

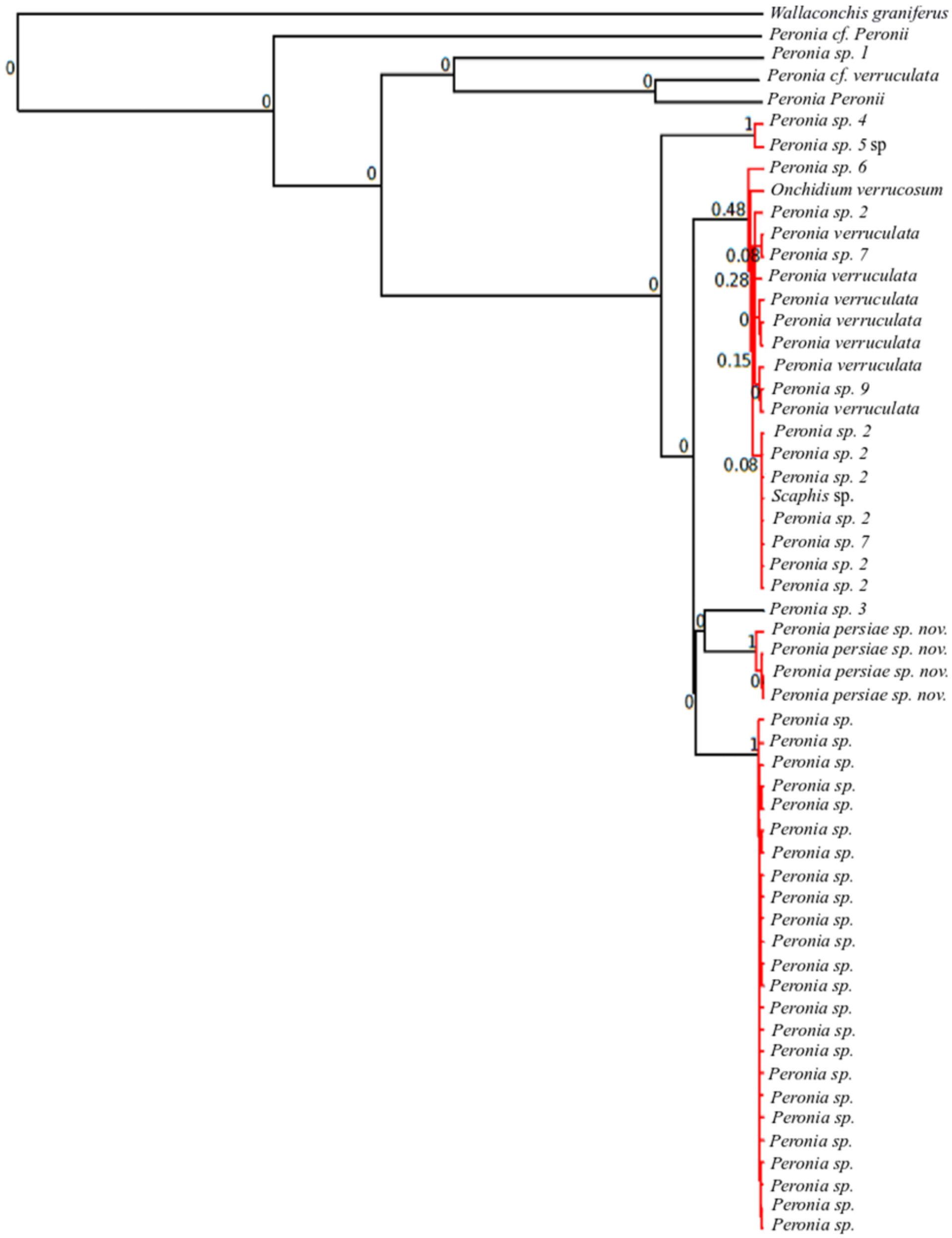

FIGURE S3. Rooted ultrametric tree from Bayesian analysis with branches coloured according to the GMYC species delimitation analysis for the genus *Peronia* based on COI alignment. Bayesian support values are shown above the branches. Black lines indicate branching processes among species; red lines indicate branching processes within species. *Wallaconchis graniferus* as outgroup and identical sequences are removed.

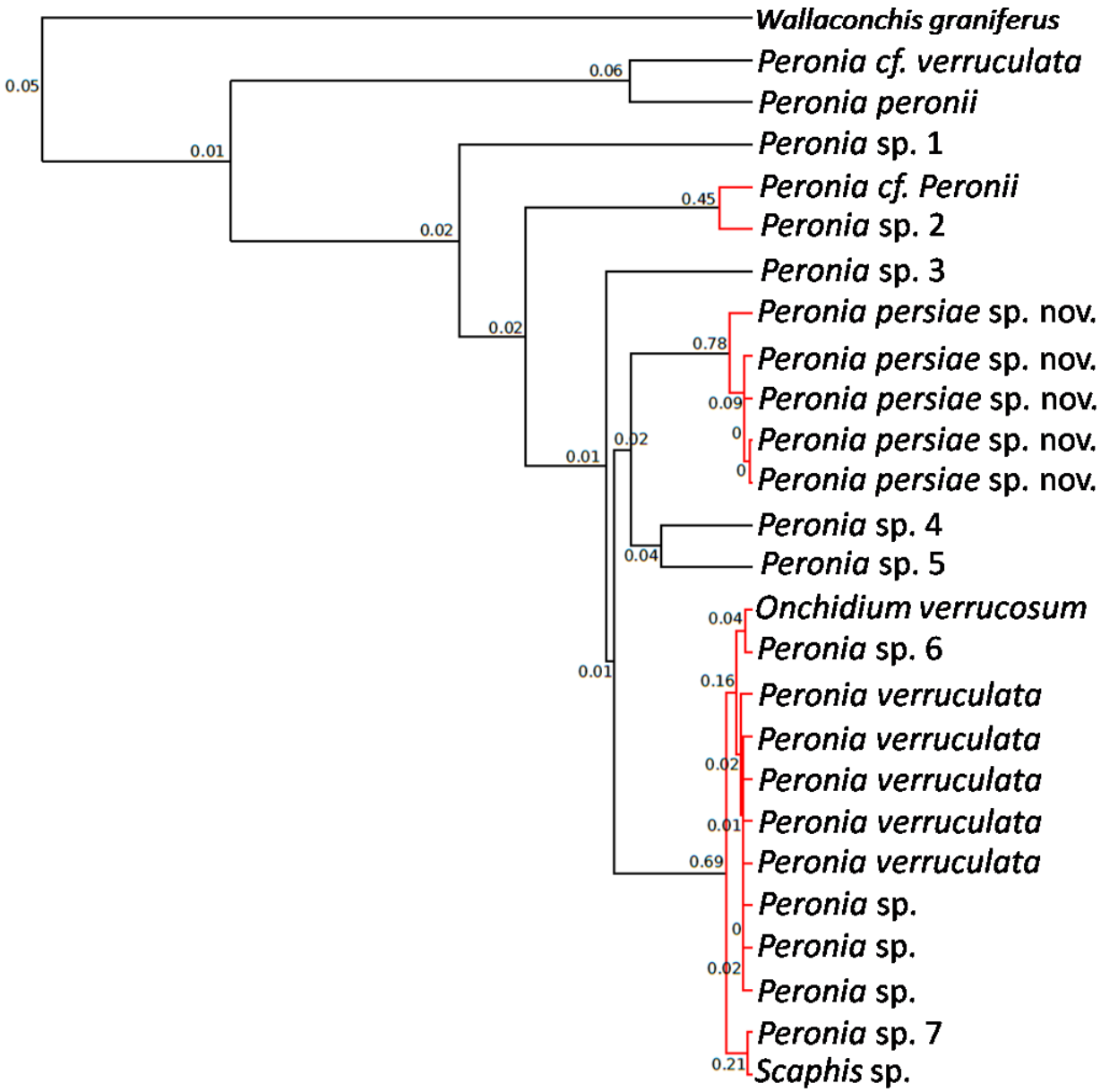

FIGURE S4. Rooted ultrametric tree from Bayesian analysis with branches coloured according to the GMYC species delimitation analysis for the genus *Peronia* based on 16S alignment. Bayesian support values are shown above the branches. Black lines indicate branching processes among species; red lines indicate branching processes within species. *Wallaconchis graniferus* as outgroup and identical sequences are removed.

Chapter 4

From Persian Gulf to Indonesia: interrelated phylogeographic distance and chemistry within the genus *Peronia* (Onchidiidae, Gastropoda, Mollusca)

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Running title: Phylogeography and chemistry of *Peronia*

Abstract

The knowledge of relationships between taxa is essential to understand and explain the chemical diversity of the respective groups. Here, 12 individuals of the panpulmonate slug *Peronia persiae* from two localities in Persian Gulf, and one animal of *P. verruculata* from Bangka Island, Indonesia, were analyzed in a phylogenetic and chemical-taxonomic framework. Based on the ABGD test and haplotype networking using COI gene sequences of *Peronia* specimens, nine well-supported clades were found. Haplotype network analysis highlighted a considerable distance between the specimens of *P. persiae* and other clades. Metabolomic analysis of both species using tandem mass spectrometry-based GNPS molecular networking revealed a large chemical diversity within *Peronia* of different clades and localities. While *P. persiae* from different localities showed a highly similar metabolome, only few identical chemical features were found across the clades. The main common metabolites in both *Peronia* species were assigned as polypropionate esters of onchitriols and ilikonapyrones, and osmoprotectant amino acid-betaine compounds. On the other hand, the isoflavonoids genistein and daidzein were exclusively detected in *P. persiae*, while cholesterol and conjugated chenodeoxycholic acids were only found in *P. verruculata*. Flavonoids, bile acids, and amino acid-betaine compounds were not reported before from Onchidiidae, some are even new for panpulmonates. Our chemical analyses indicate a close chemotaxonomic relation between phylogeographically distant *Peronia* species.

Introduction

Peronia Fleming, 1822, a panpulmonate slug genus, is typically distributed in tropical and subtropical regions of the Indo-Pacific Ocean [1]. The genus *Peronia*, with ten acknowledged species, has been reported from many localities ranging from the Indo-Pacific to the East Coast of Africa and to Hawaii [2–4]. Due to undetected cryptic speciation, species diversity is often underestimated while the widespread distributions attributed to species is often overestimated [5]. The integration of molecular and morphological methods has led to numerous discoveries of cryptic species complexes [6]. This is also true for the genus *Peronia*, as was shown by Maniei *et al.* [3].

To date, only three *Peronia* species are well investigated by molecular and/or morphology data, i.e. *P. peronii* (Cuvier, 1804), *P. verruculata* (Cuvier, 1830), and *P.* persiae Maniei, Espeland, Mohavedi & Wägele, 2020 [2, 3, 6–11]. All other species are difficult to assess due to their old and superficial descriptions based on morphological characters. Additionally, many specimens that

were just recently added to the National Center for Biotechnology Information (NCBI) have been characterized solely on the genus level as *Peronia* based on partial sequence data; an approach that does not allow for detailed species assignment relevant for all further investigations.

To analyze the evolutionary processes at the time of lineage separation, reconstruction of the genealogical relationship between genes can be studied by the use of phylogenetic trees or haplotype networks [12]. The phylogenetic approach assumes that the ancestral sequences are unobserved and associated with the internal nodes of the tree while the observed sequences are associated with its terminal nodes. In case observed sequences may be ancestral to each other, a network map approach might be more appropriate as these sequences will be associated with the internal nodes of the network. Therefore, haplotype network construction is a widely used approach for analyzing and visualizing the relationships among DNA sequences within a population or species [13]. Cytochrome oxidase subunit I (COI) gene sequence is a known DNA barcode to construct haplotype networks and to display the relationship among different geographical populations or species [13].

In addition to the genomic and morphological description, chemo-taxonomy, the characterization of species-specific metabolomes, has emerged as a complementary approach in species delineation [14]. A species and its metabolome are affected by environmental factors, being exposed to abiotic (pH, temperature, pressure, oxygen, light, salinity) and biotic factors (nutrients, presence/absence of predators) [15]. A metabolomic survey will lead to valuable insights into the lifestyle of each organism and possible species-specific communication traits that might allow for predatory/prey or defensive behavior as well as intra- and interspecific communication [16]. In this context, the family Onchidiidae, living in the intertidal flats of tropical and temperate coasts encountering high fluctuation of abiotic parameters, are of special interest. So far only polypropionate esters, cholesterol, stearic acid, ethylhexylglycerins such as chimyl alcohol, and batyl alcohol have been reported from these pulmonate slugs [17–19].

This study combines for the first time a phylogenetic and metabolomic survey of slug species belonging to the genus *Peronia*, which lives in rocky shore habitats and feeds on turf algae covering rocks [3]. In particular, the geographic relationship amongst Iranian *Peronia* haplotypes from two different localities was compared by phylogenetic reconstruction, species delimitation, and haplotype networking. Subsequently, the secretome of phylogeographical distinct species was compared to other specimens of distinct *Peronia* species widely distributed in the Indo-Pacific to identify a possible core metabolome and specific-specific traits.

Results

Species delimitation and haplotype networking of *Peronia* species

The ABGD test results on the *Peronia* COI sequences indicated nine well-supported groups in this genus, hereafter referred to as clade 1 to 9 (Figure 1). All these nine clades are also found in the haplotype network (Figure 2) and reflect a geographical separation as shown in the distribution map of the genus (Figure 3). The ABGD test revealed that specimens of *P. persiae* form a separate clade (clade 2). Thus, the specimens from two localities of the Persian Gulf (Iran), Bandar Lengeh and Lavan Island, were considered as a distinct new species.

According to our results, the hitherto unidentified specimen from Indonesia mentioned in Papu *et al*. as *Peronia* sp. a [20] and in Maniei *et al*. as *P*. sp.7 [3] was placed in the clade 4, together with *P. verruculata* specimens from China and Australia. Therefore, we preliminarily assign this specimen from Indonesia to the species *P. verruculata*.

The haplotype network analysis highlights a considerable distance between the specimens of *P. persiae* and other clades, including our Indonesian haplotype, whereas only a few mutations were observed within the same species. None of the Iranian haplotypes are observed within any other clade, indicating no connectivity to any other *Peronia* populations. Interesting in this context is clade 1, which exists of specimens exclusively from Singapore, thus showing again the restricted gene flow between populations. In contrast, clade 4 (*P. verruculata*), which is also distributed in Singapore, contains haplotypes that are similar or the same as in Oman, Indonesia, China, Philippines, and even in Australia. Therefore, *P. verruculata* seems to be more widely distributed than all other clades (Figure 3). This indicates that *P. verruculata* lives sympatric with specimens from clade 1 in Singapore, with no connectivity. The only other overlapping distribution not involving *P. verruculata* could be shown here for clade 5 with two undescribed *Peronia* specimens and a *Peronia* cf. *peronii* in Mozambique.

However, it needs to be emphasized that the number of available sequences for all other clades is very limited and further sampling is required.

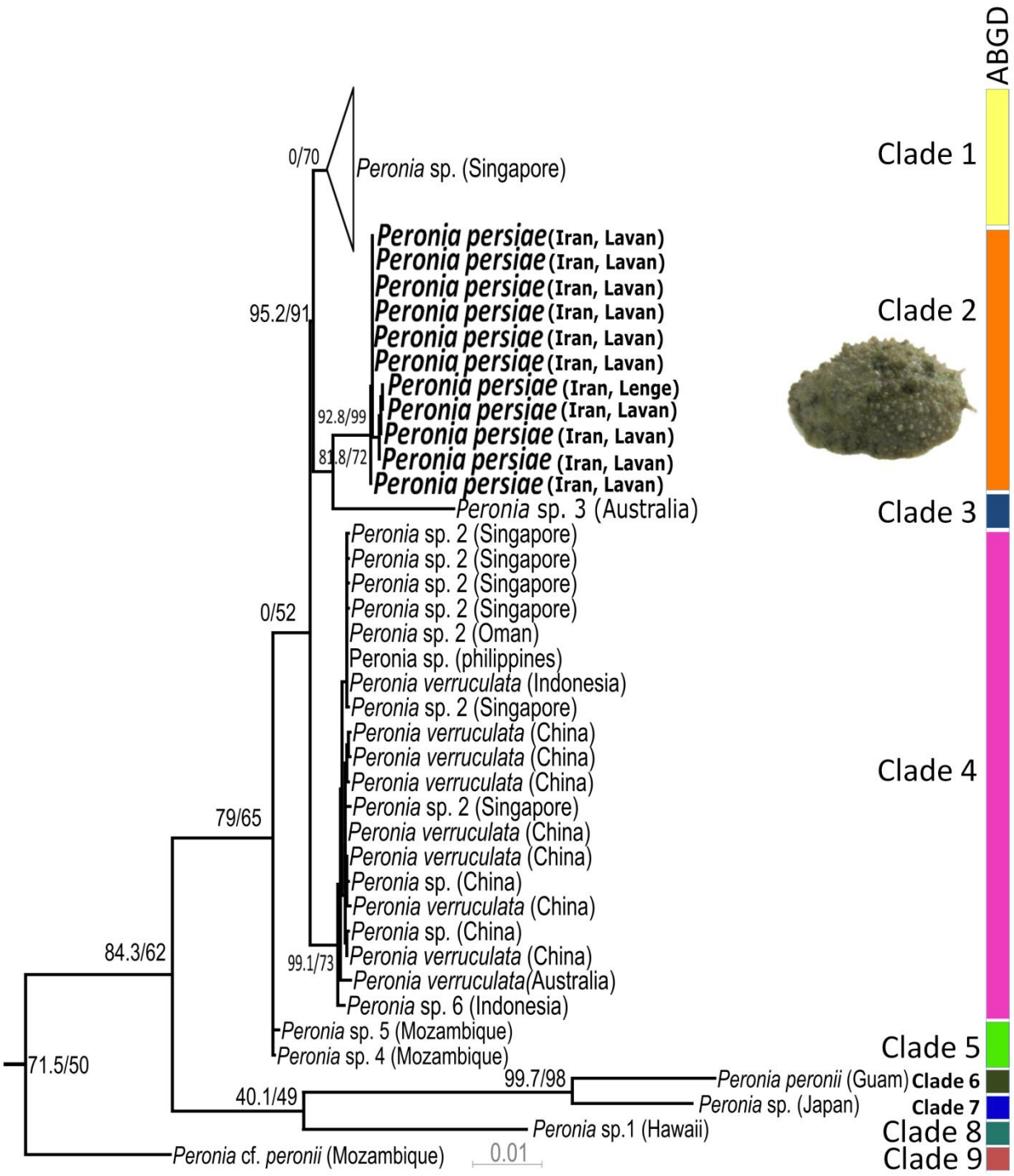

Figure 1. Maximum likelihood tree of the genus *Peronia* based on COI data set, *Wallaconchis graniferus* (Semper, 1880) used as outgroup (not shown here). The clades within the genus *Peronia* resulting from the ABGD test are visualized on the right side, using different colors for each clade. Numbers before and after slash indicate approximate likelihood ratio test (SH-aLRT)

and ultrafast bootstrap values, respectively (graph modified from Maniei *et al*. 2020). Inlay picture shows one specimen of *P. persiae.*

Molecular networking of *Peronia persiae* (clade 2) and *P. verruculata* (clade 4)

To evaluate species-specific differences as well as locality dependent differences, we explored the chemical features/compounds in two *Peronia* species of different clades as well as the same species from two different localities using MS/MS-based Global Natural Product Social (GNPS) molecular networking [21].

For this purpose, the ethanolic extracts of *P. persiae* (clade 2) from the two Iranian localities of Lavan Island (G1: eleven specimens/combined) and of Bandar Lengeh (G2: one specimen) and one specimen of *P. verruculata* (clade 4) from Bangka Island, Indonesia (G3) were analyzed by HPLC coupled with high-resolution mass spectrometry and automated fragmentation (HPLC-HRMS/MS). The resulting MS/MS data were then subjected to the GNPS molecular networking platform. The resulting molecular network revealed 557 chemical features, visualized in nodes, and based on similarities in the fragmentation patterns of parent ion *m*/*z* signals were connected using edges (Figure 4).

Nodes were color-coded according to the three geographic groups. Among the 557 detected chemical features, 106 (19%) showed an overlap of at least two of the three groups. The biggest overlap was observed between *P. persiae* from Lavan Island and Bandar Lengeh (G1 and G2 with 76 nodes: 13.5%) and only 21 features (4%) were identified in the core metabolome of all three groups (Figure 5). On the other hand, 451 nodes (81%) were detected only in one group, almost half them (213 nodes: 38%) were unique features with no similar MS spectra to other compounds (singletons) and another half (238 nodes: 43%) were connected by edges to other features or analogs in the same or other groups (cosine >0.5).

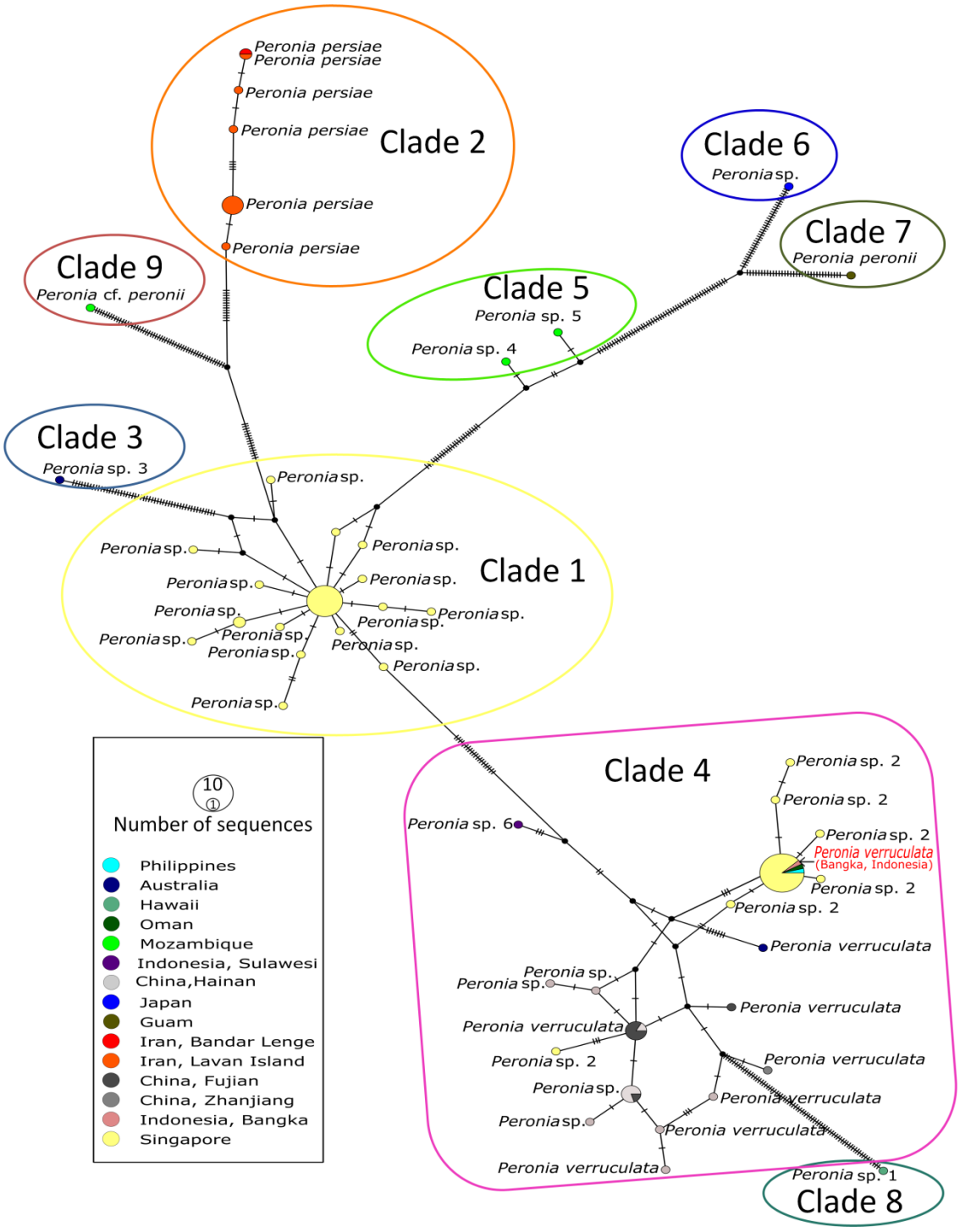

Figure 2. Haplotype network of all available *Peronia* COI sequences. Colored nodes on the haplotype network correspond to specific geographic localities, with each clade (see Figure 1) highlighted by a colored box. Hash marks on the haplotype network denote mutational steps and the size of the colored nodes corresponds to the number of sequences. *P. verruculata* from

Bangka, Indonesia (red text, clade 4), together with *P. persiae* specimens (clade 2) were used for molecular network analyses.

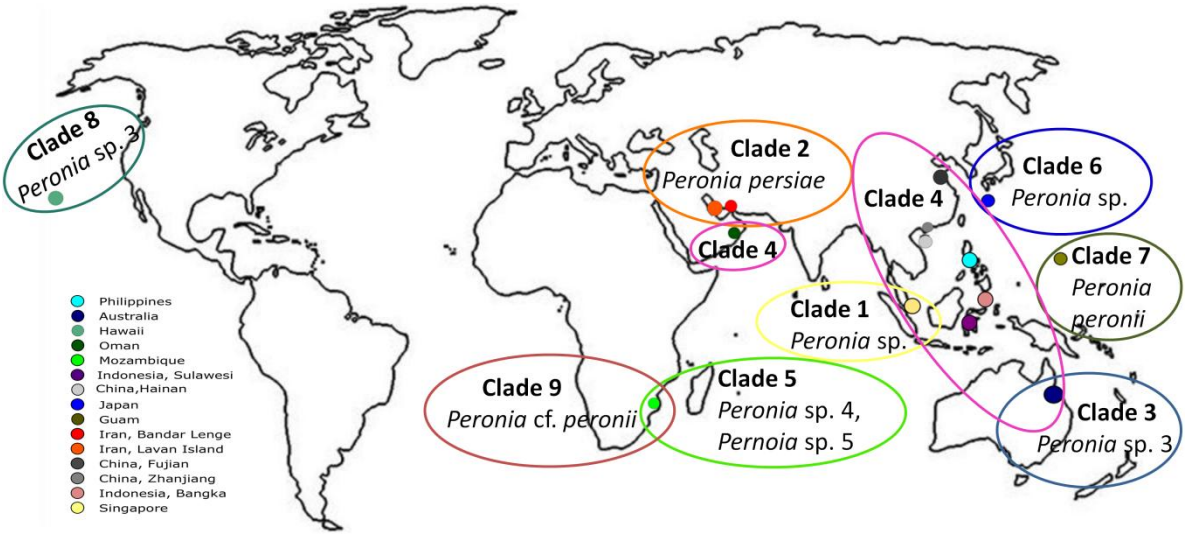

Figure 3. Geographical distribution map of *Peronia* in the Indo-Pacific Ocean with detailed localities and distribution of clades highlighted in the same color as in Figure 2. The blank thick white world map—b3c is taken from outline world map images website using the following html link: https://www.outline-world-map.com/blank-thick-white-world-map-b3c.

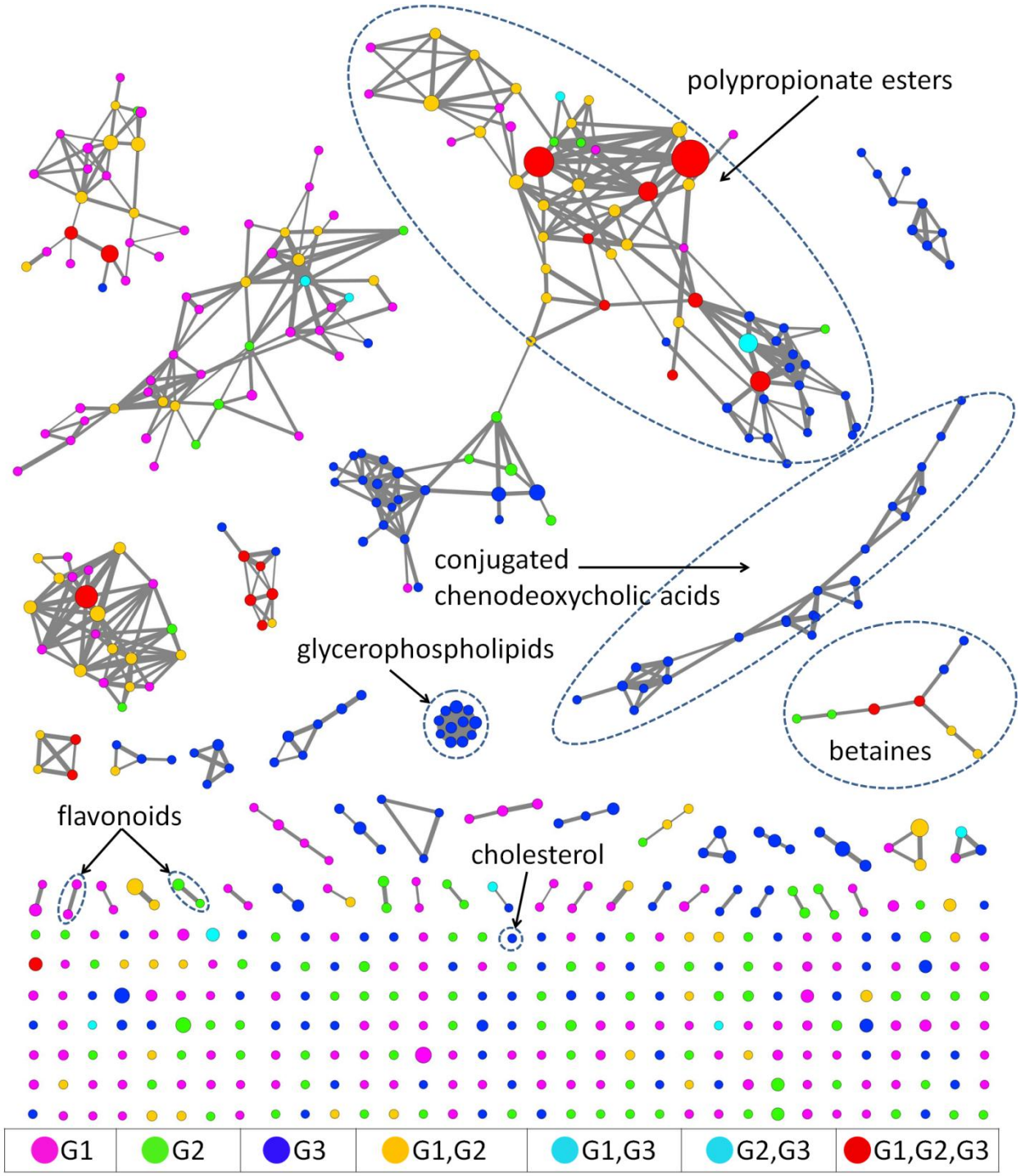

Figure 4. Molecular network of *Peronia persiae* specimens and one *P. verruculata* specimen. The network is color-coded according to detection from single or multiple groups. Dereplicated compounds are marked and the node size reflects the number of the spectra detected from each parent ion. G1: *P. persiae* from Lavan Island (eleven specimens); G2: *P. persiae* from Bandar Lengeh (one specimen); G3: *P. verruculata* from Bangka Island, Indonesia (one specimen). An interactive network is available by DOI (https://doi.org/10.18119/N9KW35).

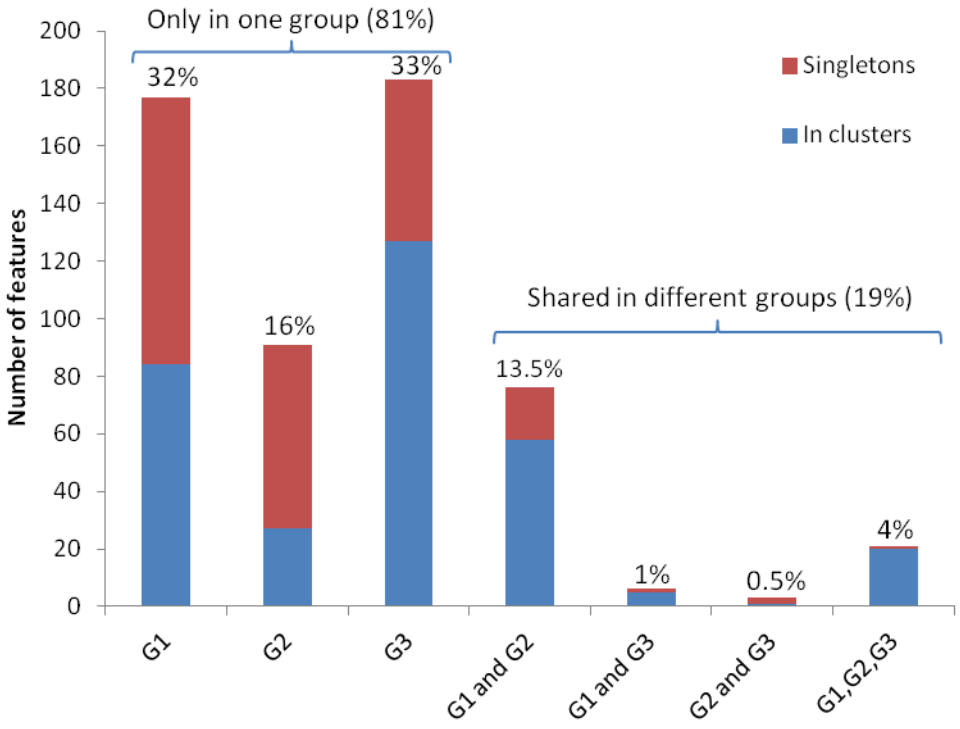

Figure 5. Contribution of the unique and shared chemical features in the molecular network. G1: *Peronia persiae* from Lavan Island (eleven specimens/combined); G2: *P. persiae* from Bandar Lengeh (one specimen); G3: *P. verruculata* from Bangka Island, Indonesia (one specimen).

In addition, a comparative analysis of the total UV absorption spectra (254 nm) of each sample (G1, G2, and G3) was pursued revealing nearly identical chromatograms of G1 and G2. Chromatogram G3, however, varied in peak counts, intensities, and retention times. In summary, several chemical features of the molecular network were putatively assigned based on their *m/z* ratio, UV absorption, and retention time using the GNPS library and the *in silico* dereplication tool Dereplicator-plus [22] (Figure 4, Table 1).

Table 1. List of chemical features putatively assigned using GNPS and Dereplicator-plus from the resulting MS/MS data of *Peronia persiae;* Lavan Island, Iran (G1), *P. persiae;* Bandar Lengeh, Iran (G2), and *P. verruculata;* Bangka Island, Indonesia (G3).

Chemical feature	***m/z***	**retention time (min)**	**detected group**
onchitriol I A	629.362 $[M+H]^+$	15	G1,G2
onchitriol I B	643.376 $[M+H]^+$	15.8	G1,G2
onchitriol I C	643.376 $[M+H]^+$	13.8	G1,G2
onchitriol I D	699.437 $[M+H]^+$	18.6	G1,G2,G3
onchitriol II B	601.368 $[M+H]^+$	14.5	G1,G2
11,13-dipropanoyl-Ilikonapyrone	657.391 $[M+H]^+$	16.5	G1,G2,G3
11-(3-methylbutanoyl)-13-propanoyl-Ilikonapyrone	685.418 $[M+H]^+$	17.6	G1,G2,G3
glycine betaine	257.147 $[2M+Na]^+$	1.1	G1,G2,G3
proline betaine	283.164 $[2M+Na]^+$	1.2	G1,G2,G3
hydroxyproline betaine	299.150 $[2M+Na]^+$	1.2	G3
genistein	271.057 $[M+H]^+$	9.2	G1
daidzein	255.063 $[M+H]^+$	7.8	G1
kaempferol-3-O-galactoside	471.090 $[M+Na]^+$	26.6	G2
cholesterol	271.057 $[M+H]^+$	12	G3
phenylalanine detagujnoc sdica cilohcyxoedonehc	1043.710 $[2M+Na]^+$	16.1	G3

As depicted in Figure 4, the cluster containing most of the detectable chemical features was putatively assigned as polypropionate esters, which were also identified from other panpulmonates [17,18]. The largest nodes/chemical features within this cluster corresponded to the main UV peaks and highest signal in the LC-MS chromatograms of all groups (Figures 6 and 7). Further analysis allowed for the tentative assignment of seven chemical features as onchitriol I A-D, onchitriol II B and two propanoyl-ilikonapyrones (Figure 7). All derivatives share similar chemical features, such as 32 carbon atoms in their backbone with two γ-pyrone rings, but differ

in the substitution of hydroxy, O-acetyl, O-propanoyl, and O-metylbutanoyl moieties on carbon 3, 13, 15 (onchitriols) or carbon 3, 11, 13 (ilikonapyrones). Onchitriols and ilikonapyrones also differ in the position of a double bond between C11–C12 or C14–C15, respectively (Figure 7).

Comparison of G1, G2, and G3 showed that the relative ratio of these compounds differed between G1/G2 to G3. The main compounds assigned in G1 and G2 were onchitriol I B (*m/z*: 643.376 $[M+H]^+$), 11,13-dipropanoyl-ilikonapyrone (*m/z*: 657.391 $[M+H]^+$), an unknown derivative (*m/z*: 671.405), and 11-(3-methylbutanoyl)-13-propanoyl-ilikonapyrone (*m/z*: 685.418 $[M+H]^+$), which all differ in 14 *m/z* units (Figures 6 and S1). In contrast, the main compounds detected in G3 could not be assigned (*m/z*: 671.405, 713.430, 727.447) (Figures 6 and S1) and relatively low intensities for 11-(3-methylbutanoyl)-13-propanoyl-ilikonapyrone (*m/z*: 685.418 $[M+H]^+$) were detectable.

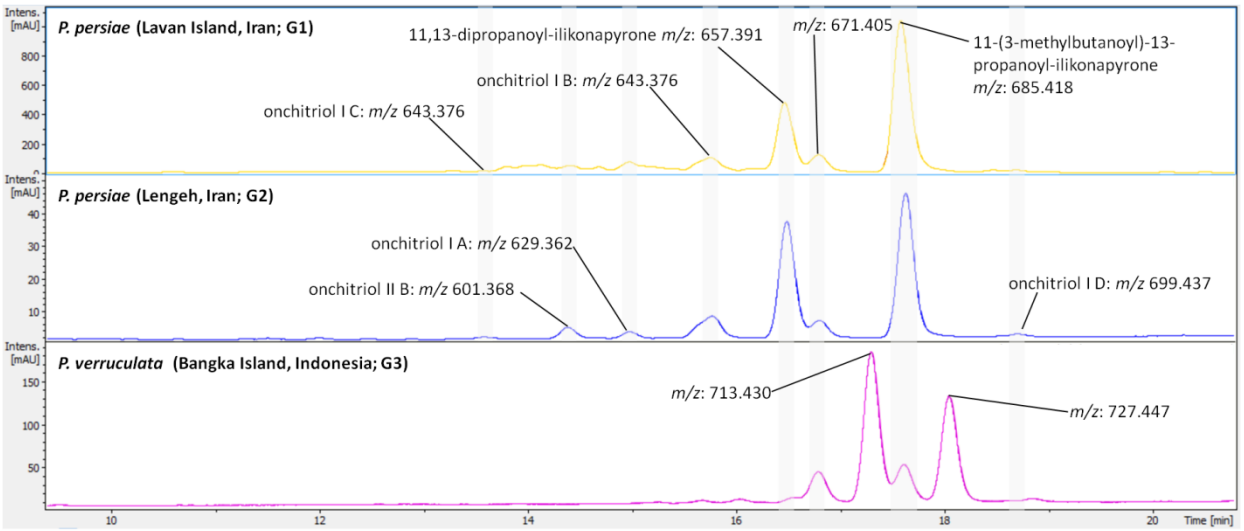

Figure 6. LC-UV (254 nm) trace of three groups of *Peronia* species extracts. Different chemical features assigned as polypropionate compounds are marked. Grey bars represent the same compounds found in different groups. MS/MS spectra are given in Figure S1.

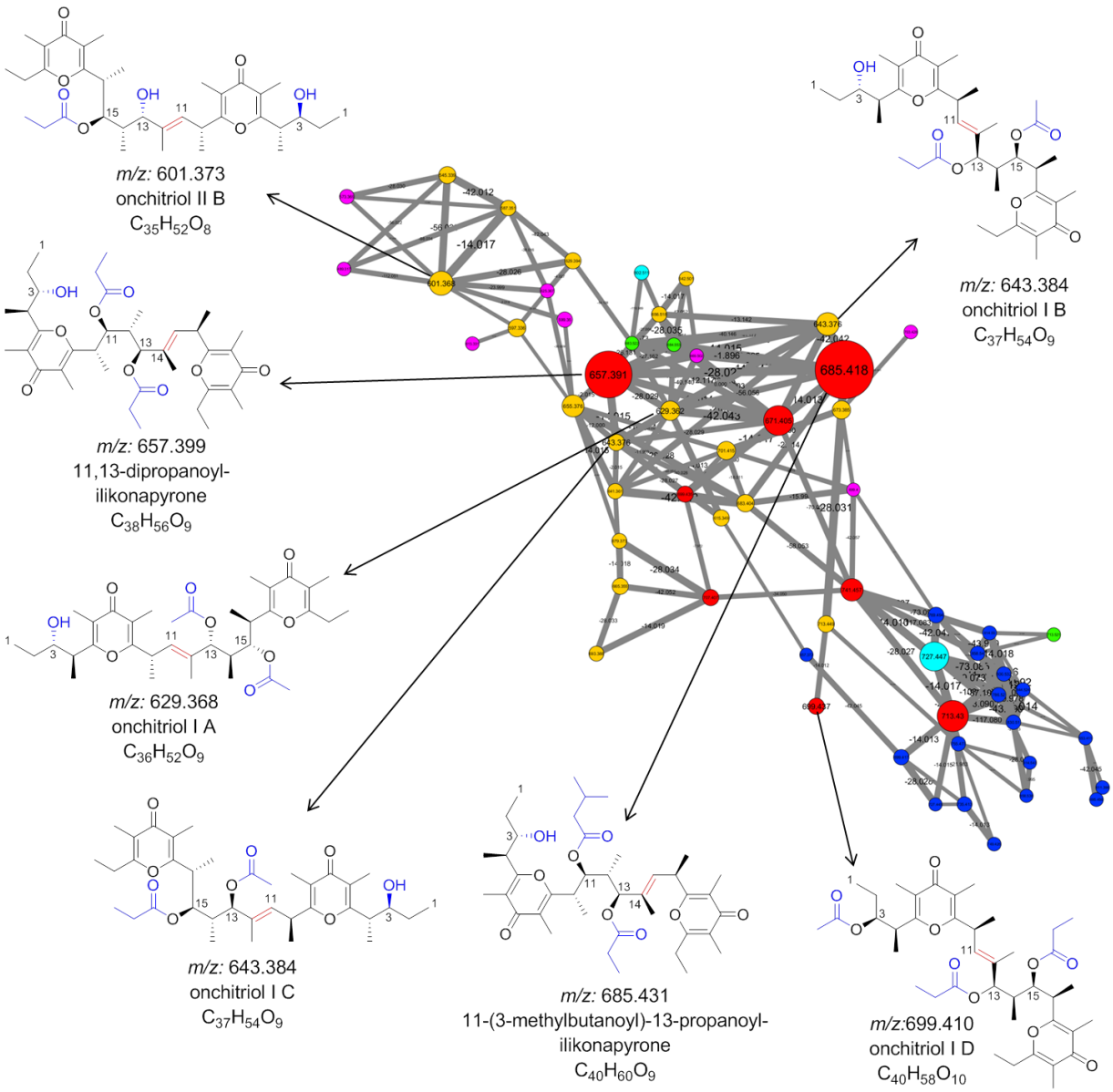

Figure 7. Dereplicated polypropionate esters in the molecular network of *Peronia persiae* and *P. verruculata* extracts. The backbone is in black and the different substitution of hydroxy, O-acetyl, O-propanoyl, and O-metylbutanoyl moieties are highlighted in blue. Indicative double bonds of onchitriols and ilikonapyrones are displayed in red. Node colors in subnetwork represent chemical features detected from different groups; pink (G1: *P. persiae* from Lavan Island, Iran), green (G2: *P. persiae* from Bandar Lengeh, Iran), dark blue (G3: *P. verruculata* from Bangka Island, Indonesia), orange (G1 and G2), light blue (G1/G2 and G3), and red (G1, G2, and G3).

Another characteristic cluster, assigned by GNPS and found in all three groups contained very hydrophilic compounds (RT: 1.1-1.2 min) belonging to the class of amino acid-betaine compounds. Glycine betaine was detected as *m/z*: 257.147 $[2M+Na]^+$ which is connected to another node with the *m/z* shift of 26.017. This corresponds to the mass of proline betaine zwitterion complex, together with glycine betaine having an *m/z* of 283.164 (Figure 8). Compounds of this class are zwitterions and contain a quaternary ammonium cation and a carboxylate anion. The MS/MS fragmentation of both complexes revealed the monomeric ions with corresponding masses of sodium adducts (Figure 8). Another node in this cluster corresponds to hydroxyproline betaine (Figure S2).

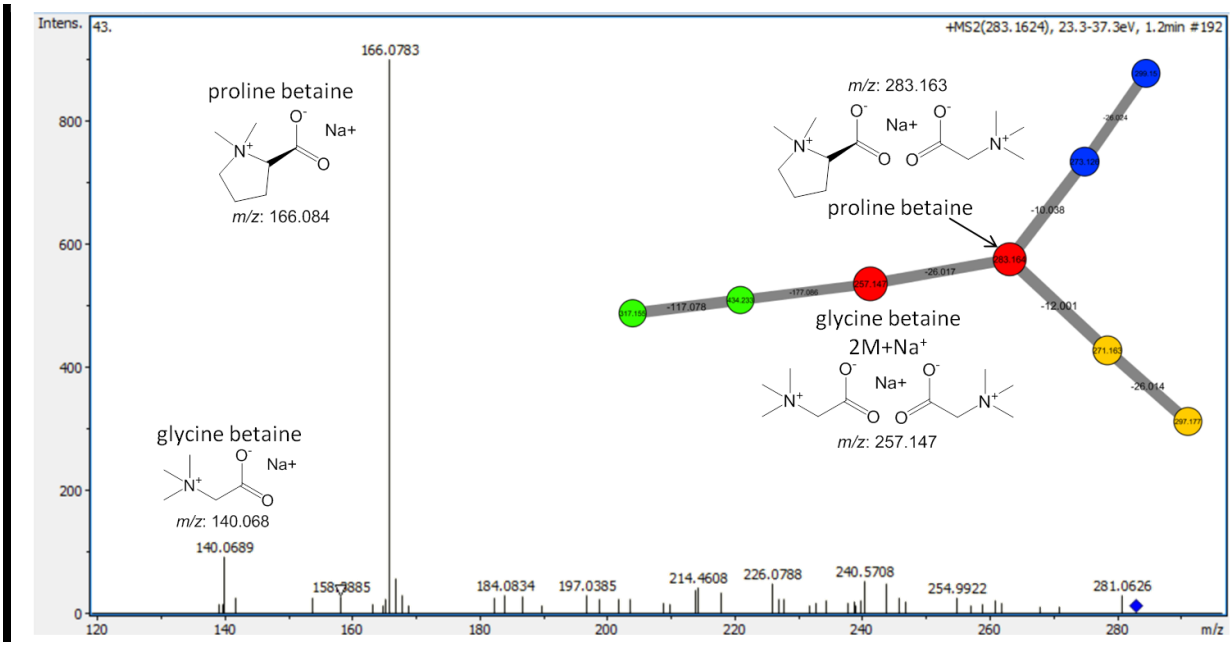

Figure 8. Amino acid-betaine cluster dereplicated from extracts of both *Peronia persiae* and *P. verruculata* with different localities. MS/MS spectra of proline betaine complex are given here and the MS/MS spectra of glycine betaine and hydroxyproline betaine are given in the supplementary data (Figure S2). Node's colors represent chemical features detected from different groups; green (G2: *P. persiae* from Bandar Lengeh, Iran), blue (G3: *P. verruculata* from Bangka Island, Indonesia), orange (G1: *P. persiae* from Lavan Island, Iran and G2), and red (G1, G2, and G3).

Furthermore, flavonoid compounds typically produced by plants [23], such as genistein (*m/z*: 271.057 $[M+H]^+$) and daidzein (*m/z*: 255.063 $[M+H]^+$), were detected solely within G1 and G2,

while the glycosylated compound kaempferol-3-O-galactoside was exclusively detected in G2 (Figures 9 and S3).

The second species-specific cluster found only in *P. verruculata* (G3) was annotated as cholesterol and conjugated chenodeoxycholic acids (Figures 9 and S4).

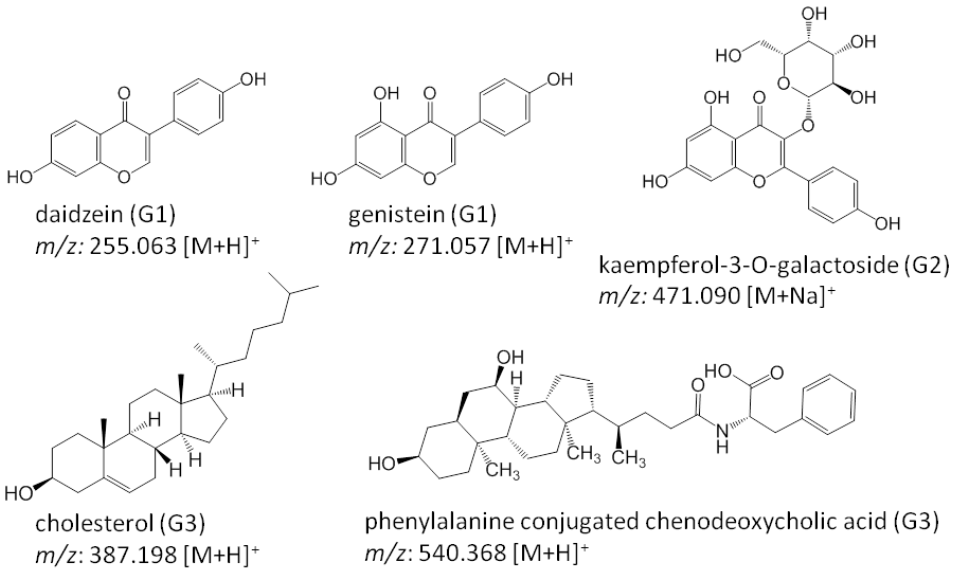

Figure 9. Unique features dereplicated only in one of the investigated *Peronia* species. G1: *P. persiae* from Lavan Island, Iran, G2: *P. persiae* from Bandar Lengeh, Iran, and G3: *P. verruculata* from Bangka Island, Indonesia. Mirror MS/MS spectra against GNPS library spectra are given in Figures S3 and S4.

Discussion

Despite many systematic and phylogenetic analyses of Onchidiidae [9,24,25], our knowledge of the species diversity and distribution, especially in the genus *Peronia*, is still rudimentary. The ABGD test of the COI gene of genus *Peronia* revealed nine well supported distinct clades and the constructed haplotype network confirms the ABGD test result. This is in line with divergence distance results in our previous study [3] and demonstrates that haplotype networking can be useful for the delimitation of putative cryptic species, even if anatomical studies are not available. The level of mutations in each clade with more than two specimens (clades 1, 2, and 4) was relatively low, whereas the distances between the clades are high. With our analysis here, we could confirm the distinctiveness of *P. persiae* (clade 2) from all other clades by haplotype network analyses.

Our metabolomic investigations also indicate its chemical distinctiveness from the second *Peronia* species investigated here, *P. verruculata* (clade 4).

In contrast to all other clades, clade 4 (*P. verruculata*) shows a very wide distribution from Oman to southern China and even Australia. Many unassigned specimens in this clade were collected from Singapore. Interestingly, clade 1 comprises only sequences from this particular locality. Chang *et al*. reported that the specimens of *Peronia* sp. from Singapore are morphologically very similar [6], while our results on genetic distance analysis clearly separate the Singapore specimens into two groups. Clade 1 is an undescribed cryptic species, whereas clade 4 includes other haplotypes, partly identified as *P. verruculata*. *Peronia verruculata* from Bandra in India was described morphologically in great detail by Awati and Karandikar [2]. All available *P. verruculata* haplotypes used in our analysis have not been described morphologically. Therefore, the assignment of all other unnamed *Peronia* haplotypes within the clade 4, including our sequence from Indonesia, to *P. verruculata* should be regarded as preliminary until a confirmed reference sequence of this species is available.

The distribution of the intertidal fauna and flora are greatly affected by hydrographical conditions [26]. This certainly also holds for members of the intertidal living Onchidiidae. The habitat shifts and changes in abiotic factors such as salinity, temperature, oxygen level, and light intensity towards higher extremes have affected the evolution and radiation of the panpulmonate molluscs [27, 28, 29]. Our result on the chemical composition of *P. persiae* from different localities in the Gulf (Lavan Island vs. Bandar Lengeh) that generally encounter higher temperature and salinity values [3] showed a similar mixture of the main chemical features in their body extracts, thus indicating no regional differences within this species. However, the chemical composition was different from the *P. verruculata* specimen from Indonesia.

Common secondary metabolites detected within all *P. persiae* specimens were the flavonoids genistein and daidzein, with the glycosylated form only found in the single specimen from Bandar Lengeh. These compounds are widely distributed in plants and are known to be potent antioxidants. Therefore, these metabolites may help slugs in the intertidal zone with a high level of oxidative stress induced by desiccation during low tides [30, 31]. Indeed, genistein showed antimutagenic effects with DNA damage agents at a concentration of 10 μM [32]. A similar bioactive flavonoid, apigenin, was also isolated from the intertidal aplysiid heterobranch *Syphonota geographica* (A. Adams & Reeve, 1850) (Aplysiidae, Tectipleura) and its food, the seagrass *Halophila stipulacea* (Forsskål) Ascherson, 1867 [33].

These findings are indicative for the food dependency of *P. persiae* for these flavonoids as members of this genus are found grazing on intertidal algae-covered rocks [1]. Secondary metabolites of dietary origin are known to be modified by the sea slugs, providing them a wider range of ecological opportunities [33]. taht detaluceps eb dluoc ti ereH *P. persiae* either feeds and/or accumulates plant-derived metabolites for defensive purpose granting them a better survival probability.

In contrast, cholesterol and *bile acids* were exclusively identified from *P. verruculata.* Bile acids are water-soluble steroids formed during the catabolism of cholesterol to primary bile acids, i.e. cholic acids and chenodeoxycholic acids [34] and show anticancer and antibacterial activities [35,36]. Cholesterol was previously reported from *Onchidium reevesii* (Gray, 1850) [17], octocorals [37], and sponges [38], while there are only a few reports of bile acids and derivatives in other marine invertebrates. Bile acid derivatives in marine invertebrates are considered to be of symbiotic microbial origin [39]. In this case, *P. verruculata* should have a symbiotic bacterial strain that produces the bile acids which then can be conjugated with different amino acids to provide chemical defense for the host, an interesting hypothesis that has to be proven yet.

Compounds that were found in both species also included a diverse mixture of polypropionate esters as the most common and prominent chemical features. Polypropionates are reported from various Onchidiidae, including *Onchidium reevesii* [17], partly described under the former name *Onchidium struma* (nomen nudum) [40], various unidentified *Onchidium* species [18, 19, 41], and also *P. verruculata* [42]. Polypropionates with different carbon skeleton were also described from genus *Siphonaria* (Gastropoda: Tectipleura) such as *Siphonaria baconi* Reeve, 1856 (now accepted as *Siphonaria zelandica* Quoy & Gaimard, 1833) [43], and *Siphonaria diemenensis* Quoy & Gaimard, 1833 [44]. Moreover, other marine molluscs, like photosynthetic members of the order Sacoglossa, harbor a big complex of different polypropionates acting as photoprotectants and antioxidants within the slugs [45, 46]. These compounds seem to be common within the Onchidiidae and are reported from various localities, including China, Hawaii, and Australia [18, 44, 47]. However, the diversity and contribution of these metabolites with respect to each species was not reported before. Onchitriol and Ilikonapyrone compounds are polypropionates whose skeletons contain 32 carbon atoms, two γ-pyrone rings, and several hydroxy groups [19]. They were previously isolated from *P. verruculata* (as *Onchidium verruculatum*) [42], and also from other members assigned to the genus *Onchidium* (as *Onchidium* sp., *Onchidium* sp.1, and *Onchidium* sp.2) [18,41]. The identity

of these species, even genus affiliation, has not yet been fully verified and the assignment should be considered with caution.

Onchitriol and Ilikonapyrone compounds were detected during our study in *P. persiae* and *P. verruculata*, although, the two species showed different ratios and types of these polypropionate compounds in their body extracts. Other polypropionate compounds such as onchidiol [48,49], onchidionol [49] and onchidione [49,50] from *Onchidium* sp., and peroniatriols from *P. peronii* [51] could not be dereplicated from the here investigated species. A common polyketide biosynthetic origin in panpulmonates is proposed for polypropionate metabolites, and the diversity of these compounds can arise when the respective polyketide chain precursors folded in a different manner [44, 47]. These metabolites are shown to repel predators such as shrimps, with a minimum effective dose of 1.0 mg/mL [50]. Therefore, the major presence of these lipophilic metabolites in *Peronia*, and also other onchidiid species suggests their involvement in the chemical defense of the slugs as deterrence agents against predators. Additionally, interesting antitumor, anticancer, as well as antiviral activities have been shown from some of these polypropionate compounds [18, 19, 52].

An additional important compounds cluster detected in both *P. persiae* and *P. verruculata* has been assigned to the hydrophilic zwitterionic amino acid-betaine compounds. They are major osmolytes in response to hyperosmotic conditions that accumulate in different concentrations during the stress time. Therefore, they have a critical role during the low tide in the presence of evaporation and increased salinity. Indeed, proline betaine was reported as an effective osmolyte in the extremely euryhaline sea slug, *Elysia chlorotica* Gould, 1870 (Sacoglossa, Gastropoda) [53].

To the best of our knowledge, the dereplicated flavonoids, bile acids, and amino acid-betaine compounds were not reported from *Onchidiidae* before. However, similar flavonoids from *Syphonota geographica* [33], betaines from *Elysia chlorotica* [53], and bile acid derivatives from octocorals [37] and sponges [38] were previously reported from other sea slugs or marine invertebrates.

To conclude, our investigation of the genus *Peronia* revealed nine distinct clades of COI gene sequences, with *P. persiae* forming a chemically distinct species, only distributed in the Persian Gulf. All other clades, except *P. verruculata*, showed a geographically narrow distribution. Molecular networking revealed a large chemical diversity including compounds, which have not

been reported from Onchidiidae, or even panpulmonata before. Species or geographically related compounds are antioxidant flavonoids, found only in *P. persiae,* and cholesterol and its derived conjugated chenodeoxycholic acids in *P. verruculata*. A major common group of food deterrent compounds shared by both species comprises polypropionates, which differed only in type and ratio between *P. persiae* and *P. verruculata*, as well as the osmoprotectant amino acid-betaine compounds important to cope with osmotic stress. In how far all the other clades are characteristic in their chemical composition and the value of chemotaxonomy remains to be investigated for all other cryptic and undescribed species, as well as those *Peronia* species, for which no molecular barcodes are available at the moment.

Materials and Methods

P. persiae specimens were collected in the intertidal zone during low tide from the surface of rock or rock crevices at Bandar Lengeh, Iran (26°33′29″N 54°52′50″E) in March 2015 (one specimen), and Lavan Island, Iran (26°48′20.99″N 53°16′4.80″E) in February 2016 (11 specimens). One specimen of *P. verruculata* (assigned as *Peronia* sp. a in Papu *et al*. 2020 and as *Peronia* sp. 7 in Maniei *et al*. 2020) was collected at Bangka Island, Indonesia (2° 15′ 0″ S, 106° 0′ 0″ E) in September 2018 (Table S1). All specimens were 4-6 cm in length and preserved in EtOH 96%. Small pieces of the foot were used for molecular barcoding, and the preservation alcohol was used for metabolomic experiments. Sequenced specimens of *P. persiae* are deposited as a voucher at the Zoologische Staatssammlung München, Germany. The specimen *P. verruculata* is part of the reference collection of Sam Ratulangi University, Manado, Indonesia and was kindly provided by A. Papu.

DNA extraction, PCR, and DNA sequencing

DNA isolation was carried out using the Qiagen DNeasy Blood and Tissue kit, following the manufacturer's instructions. Partial sequences of mitochondrial COI (ca. 680 bp) were amplified by polymerase chain reaction (PCR) using the primers LCOI490-JJ (5' – CHACWAAYCATAAAGATATYGG-3') and HCO2198-JJ (5'-AWACTTCVGGRTGVCCAAARAATCA-3') [54] for COI; 16Sar-L (5′ - CGCCTGTTTATCAAAAACAT-3'). The following thermoprofile during the PCR was used: 15 min at 95 °C; 40 cycles with following reaction conditions were involved: initial denaturation at 94 °C for 35 sec, subsequent annealing at 55 °C for 90 sec, elongation at 72 °C for 90 sec and final elongation step of 72 °C for 10 min. Sequencing was performed by Macrogen Europe

(Amsterdam, Netherlands). Sequences are deposited in GenBank with the accession numbers listed in Table S2.

Phylogenetic reconstruction

Peronia sequences were downloaded from NCBI, and identical sequences removed. The final alignment contained 157 sequences including eleven sequences of *P. persiae* from both localities and the one sequence of *P. verruculata* from Bangka Island (see table S2) with two sequences of *Wallaconchis graniferus* as outgroup.

Sequences were edited using BioEdit (ver.7.2.6.1) [55] and aligned using MAFFT [56] in Geneious v7.1.9 [57]. The maximum likelihood (ML) analysis was performed in IQ-TREE [58,59], using the online version 1.6.3 on a web server (http://iqtree.cibiv.univie.ac.at/). The evolutionary model GTR was applied. Support values were calculated based on 1000 ultrafast bootstrap replicates and the approximate likelihood ratio test (SH-aLRT) (2000 replicates) was applied. Dendroscope (version 3.5.8) [60] and Inkscape (version 0.92) (https://inkscape.org/en/) were used to edit the phylogram.

Species delimitation. The Automatic Barcode Gap Discovery test (ABGD) [61] was applied for delimiting the species within this CO1 data set using default values and the evolutionary model Kimura K80. This test is independent of predefined species groups [61,62].

Haplotype networking

A statistical parsimony analysis [63] was conducted with all individual COI sequences from the 157 CO1 sequences containing data set already used for the phylogenetic reconstruction and species delimitation test, using the program TCS v.1.21 [64] in PopART [65] with a 95% connection limit and 5000 iterations. This program helps to identify haplotypes that were shared among individuals, and also calculates the number of substitutions connecting haplotypes in the network (Templeton *et al*. 1992). The program also allows for including and visualizing geographic information within the network.

Metabolite extraction and HPLC-MS/MS analysis

The preservation alcohol of all eleven *P. persiae* specimens from Lavan Island, Iran was combined and subsequently analyzed for chemical composition (Group 1 or G1). The preservation alcohol of the single specimen of *P. persiae* from Bandar Lengeh, Iran was analyzed separately (G2), as was the one specimen of *P. verruculata* from Bangka Island, Indonesia (G3). The EtOH of each group was evaporated under vacuum conditions, the residue was re-dissolved

in 100 µL methanol and analyzed by a micrOTOF-QIII mass spectrometer (Bruker) with ESI-source coupled with an HPLC Dionex Ultimate 3000 (Thermo Scientific) using an EC10/2 Nucleoshell C18 2.7 µm column (Macherey-Nagel). The column temperature was 25 °C. MS data were acquired over a range from 100-3000 *m/z* in positive mode. Auto MS/MS fragmentation was achieved with rising collision energy (35-50 keV over a gradient from 500-2000 *m/z*) with a frequency of 4 Hz for all ions over a threshold of 100. HPLC begins with 90 % H_2O containing 0.1% acetic acid. The gradient starts after 1 min to 100% acetonitrile (0.1% acetic acid) in 20 min. 5 µl of a 1 mg/ml sample solution (MeOH) was injected into a flow of 0.3 ml/min [66].

Molecular networking

All MS/MS data of each group (G1, G2, and G3) were converted to mzXML format and transferred to the Global Natural Product Social Molecular Networking (GNPS) server (gnps.ucsd.edu) [21] and the molecular network was created by the online workflow at GNPS [21] using the spectra with a minimum of four fragment ions and by merging all identical spectra into nodes, representing parent masses. Compounds with similar fragmentation patterns are connected by edges, displaying molecular families with similar structural features. The data were filtered by removing all MS/MS peaks within +/- 17 Da of the precursor *m/z*. MS/MS spectra were window filtered by choosing only the top 6 peaks in the +/- 50 Da window throughout the spectrum. The resulting data were then clustered by MS-Cluster with a parent mass tolerance of 0.02 Da and an MS/MS fragment ion tolerance of 0.02 Da to create consensus spectra. Further, consensus spectra that contained less than 2 spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.5 and more than 4 matched peaks. Further edges between two nodes were kept into the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against GNPS spectral libraries. The library spectra were filtered in the same manner as the input data including analog search. All matches kept between network spectra and library spectra were required to have a score above 0.5 and at least four matched peaks. Furthermore, DEREPLICATOR plus was used for *in silico* identification of both peptidic and non-peptidic natural products [22]. The network was visualized via Cytoscape 3.6.1. The molecular network file is available at the NDEx site [67] (https://doi.org/10.18119/N9KW35).

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Author contributions

H.W. and G.M.K. designed the experiments. F.M., J.A.M., M.C., and C.B. performed the experiments and/or analyzed the data. F.M. and J.A.M. wrote the paper and all authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Supplementary information:

Table S1. Specimens used in this study. Abbreviations of locality: BL = Bandar Lengeh (Iran); LA = Lavan Island (Iran); BA = Bangka Island (Indonesia)

Specimen	**Preservation**	**Purpose**	**Length of preserved animal (mm)**
BL 1, *P. persiae*	ETOH 96%	Haplotype network, chemistry	37
LA 1, *P. persiae*	ETOH 96%	Haplotype network, chemistry	32
LA 2, *P. persiae*	ETOH 96%	Haplotype network, chemistry	22
LA 3, *P. persiae*	ETOH 96%	Haplotype network, chemistry	26
LA 4, *P. persiae*	ETOH 96%	Haplotype network, chemistry	28
LA 5, *P. persiae*	ETOH 96%	Haplotype network, chemistry	34
LA 6, *P. persiae*	ETOH 96%	Haplotype network, chemistry	32
LA 7, *P. persiae*	ETOH 96%	Haplotype network, chemistry	35
LA 8, *P. persiae*	ETOH 96%	Haplotype network, chemistry	25
LA 9, *P. persiae*	ETOH 96%	Haplotype network, chemistry	22
LA 10 *P. persiae*	ETOH 96%	Haplotype network, chemistry	13
LA 12 *P. persiae*	ETOH 96%	Chemistry	31
BA *P. verruculata* (*Peronia* sp. a in Papu *et al*. 2020/ *Peronia* sp7 in Maniei *et al*. 2020)	ETOH 96%	Haplotype network, chemistry	25

Table S2. Specimens sequenced for this study and sequences obtained from GenBank, including locality, GenBank accession numbers, and geographical coordination.

Confirmed name of species	**Locality**	**GenBank COI**	**Coordinate/ longitude**	**Coordinate/ latitude**
Peronia sp.	Philippines	HQ660050	122.96777353	9.785934
Peronia verruculata	Australia, Queensland	EF489391	142.702796	-20.917574
Peronia sp. 1	Hawaii	HQ660038	-155.582782	19.896766
Peronia sp. 2	Oman	HQ660044	55.975413	21.473533
Peronia sp. 3	Australia, Queensland	HQ660048	142.702796	-20.917574
Peronia sp. 4	Mozambique	HQ660045	34.187957	-24.916742
Peronia sp. 5	Mozambique	HQ660047	34.187957	-24.916742
Peronia sp. 6	Indonesia, Sulawesi	HQ660046	120.5279	-1.8479
Peronia sp.	China, Hainan.	HQ285979	110.349229	20.017378
Peronia sp.	China, Hainan	HQ285980	110.349229	20.017378
Peronia sp.	China, Hainan	HQ285981	110.349229	20.017378
Peronia sp.	China, Hainan.	JN543165	110.349229	20.017378
Peronia sp.	Japan, Okinawa	HQ660043	127.680932	26.212401
Peronia peronii	Guam	HQ660041	144.766159	13.433168
Peronia cf. *peronii*	Mozambique	HQ660042	34.187957	-24.916742

Peronia verruculata	China, Fujian	GU166566	120.216978	27.324479
Peronia atalucurrev	China, Fujian	GU166564	120.216978	27.324479
Peronia atalucurrev	China, Fujian	GU166563	120.216978	27.324479
Peronia atalucurrev	China, Hainan	GU166561	110.349229	20.017378
Peronia atalucurrev	China, Hainan	GU166559	110.349229	20.017378
Peronia atalucurrev	China, Hainan	GU166558	110.349229	20.017378
Peronia atalucurrev	China, Hainan	GU166562	110.349229	20.017378
Peronia atalucurrev	China, Hainan	GU166560	110.349229	20.017378
Peronia atalucurrev	China, Fujian	GU166565	120.216978	27.324479
Peronia atalucurrev	China, Hainan	GU166557	110.349229	20.017378
Peronia atalucurrev	China, Fujian	JN543154	120.216978	27.324479
Peronia atalucurrev	China, Hainan	JN543153	110.349229	20.017378
Peronia	China,	JN543152	110.359368	21.270702

atalucurrev	Zhanjiang			
Peronia persiae	Iran, Bandar lengeh	MK312167	54.888679	26.562787
	Iran, Lavan Island	MK993386-MK993395	53.268	26.805831
Peronia verruculata (*Peronia* sp. a in Papu *et al*. 2020/ *Peronia* sp7 in Maniei *et al*. 2020)	Indonesia, Bangka	MK993397	125.153055	1.79666666
Peronia sp.	Singapore	MH002607	103.851959	1.290270
		MH002590		
		MH002605		
		MH002603		
		MH002600		
		MH002599		
		MH002594		
		MH002591		
		MH002589		
		MH002586		
		MH002585		
		MH002580-		

		MH002582		
		MH002575-MH002578		
		MH002592		
		MH002596		
		MK142731		
		MK142730		
		MK142725-MK142728		
		MK142720-MK142723		
		MK142717		
		MK142715		
		MK142714		
		MK142712		
		MK142704		
		MK142706-MK142709		
		MH002597		
		MH002604		
		MH002579		
		MH002598		

		MH002602 MH002583 MK142710 MH002593 MK142729 MK142713 MH002595 MK142732 MK142724 MK142716 MH002588 MK142705 MK142719 MK142711 MH002584 MH002606 MH002601 MK142718		
Peronia sp. 2	Singapore	MK142680-MK142703 MH002574	103.851959	1.290270

		MH002573		
		MH002569		
		MH002567		
		MH002566		
		MH002559-MH002563		
		MH002548-MH002556		
		MH002546		
		MH002545		
		MH002571		
		MH002572		
		MH002547		
		MK142694		
		MK142689		
		MH002564		
		MH002550		
		MH002557-MH002558		
		MH002570		

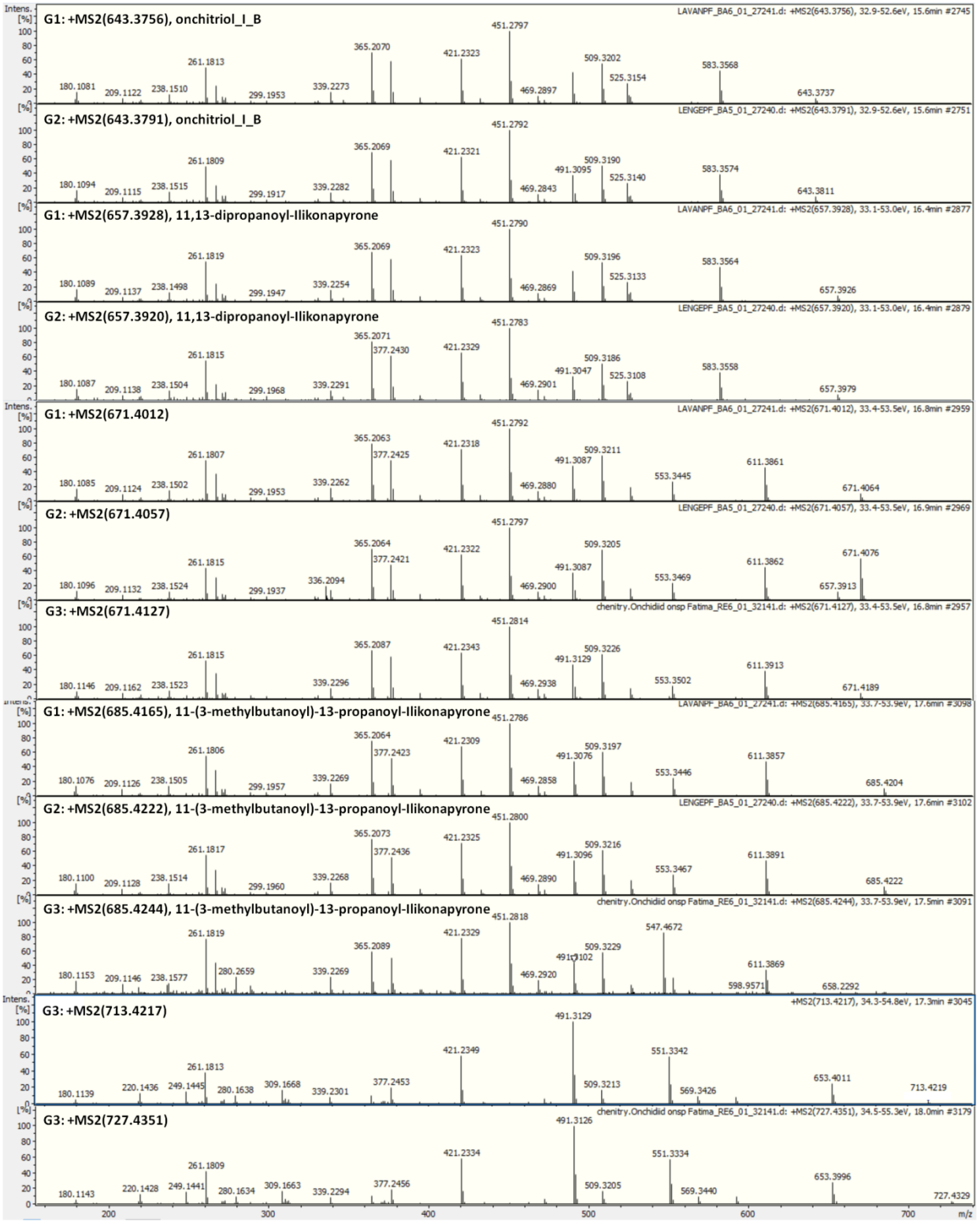

Figure S1. MS/MS spectra of the main polypropionate compounds in three groups of *Peronia* extracts. G1: *P. persiae* (Lavan Island, Iran), G2: *P. persiae* (Bandar Lengeh, Iran), and G3: *P. verruculata* (Bangka Island, Indonesia).

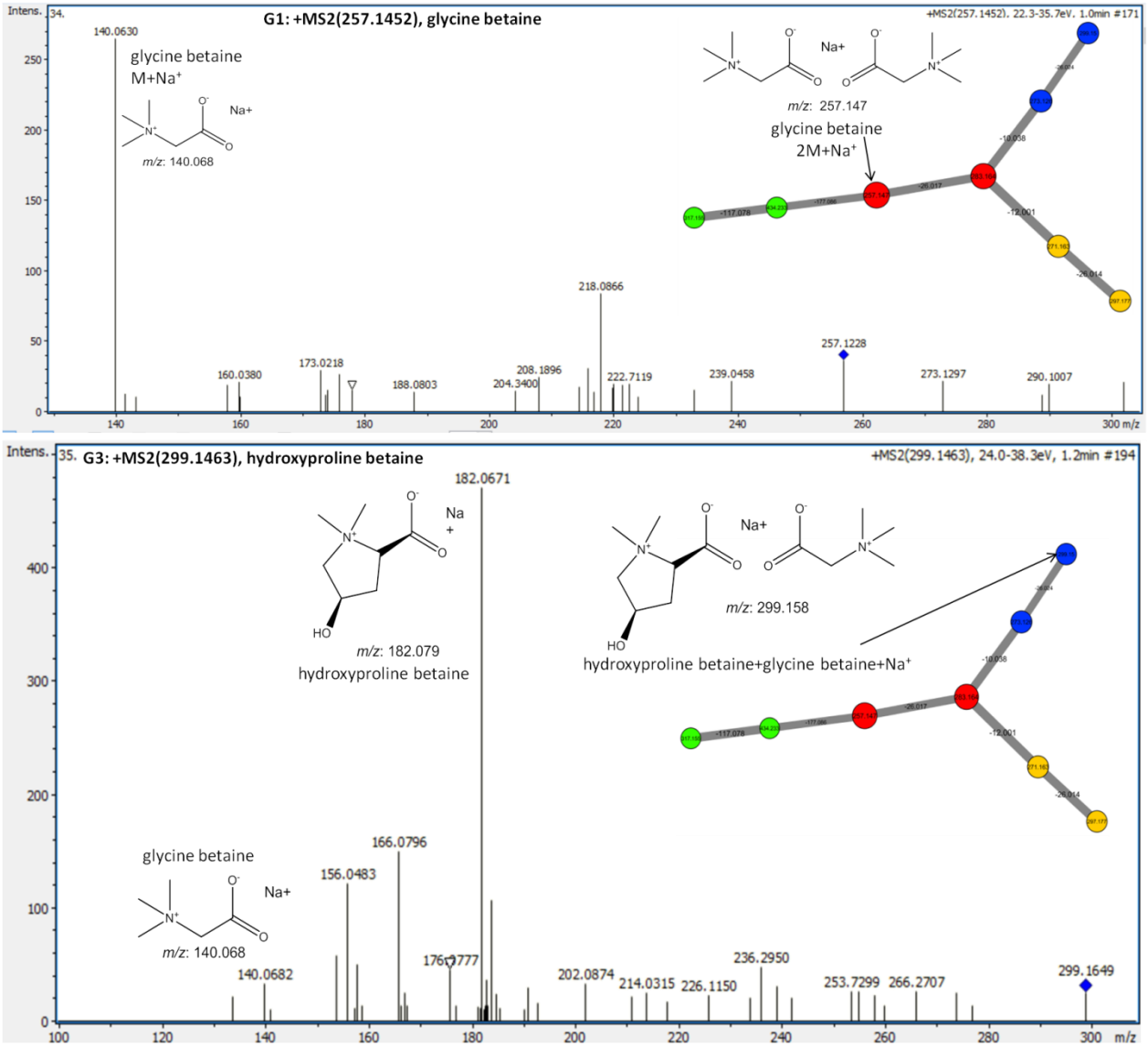

Figure S2. MS/MS spectra of glycine betaine in *Peronia persiae* and hydroxyproline betaine in *P. verruculata*.

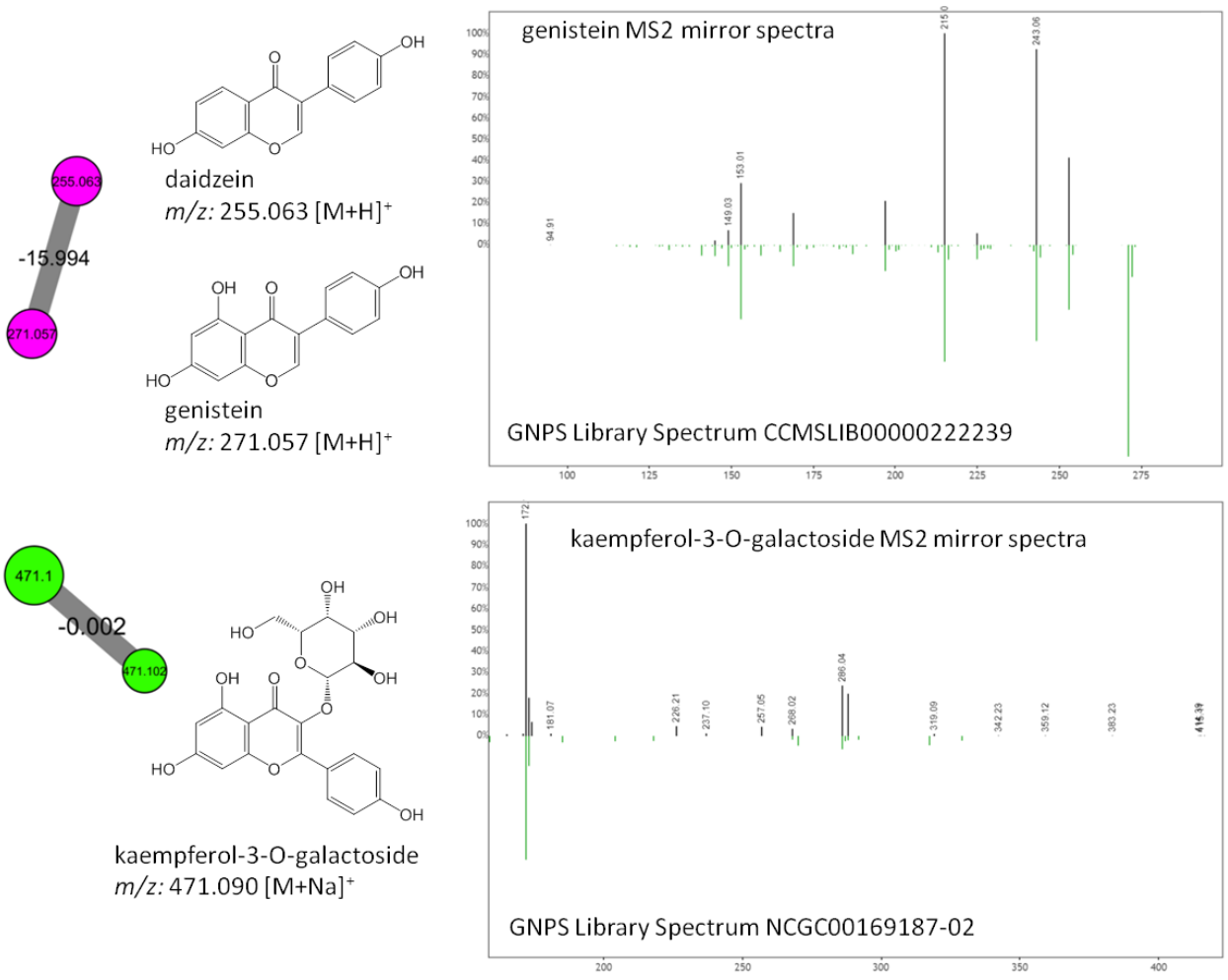

Figure S3. Flavonoid compounds dereplicated from *Peronia persiae* from different localities.

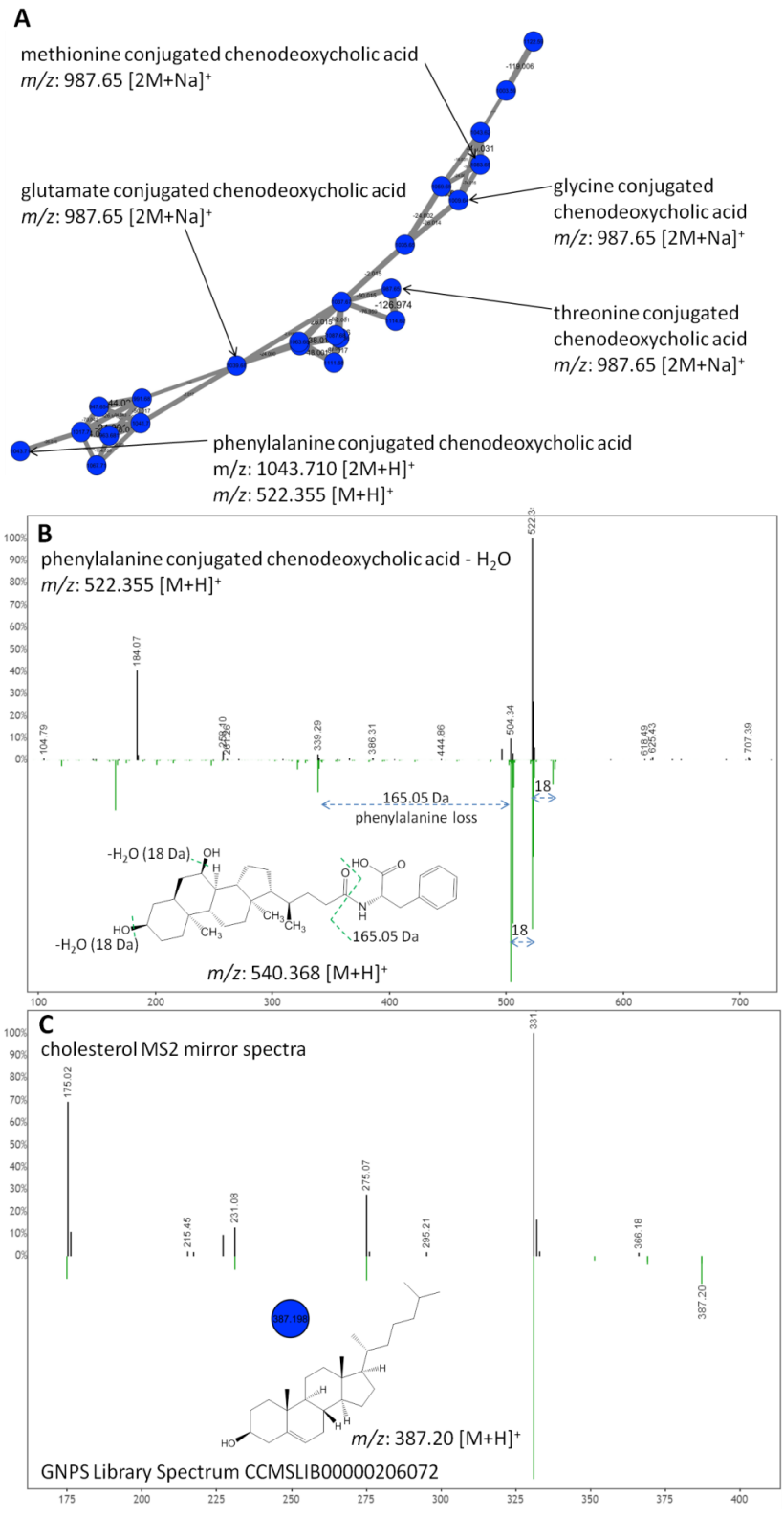

Figure S4. Dereplicated conjugated chenodeoxycholic acid cluster and cholesterol found in *Peronia verruculata*.

Chapter 5

Dendrodorididae (Heterobranchia: Nudibranchia) from Iran, Persian Gulf, with a description of a new *Doriopsilla* species.

Dendrodorididae (Heterobranchia: Nudibranchia) from Iran, Persian Gulf, with a description of a new *Doriopsilla* species

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Running title: DNA barcoding and histology of Dendrodorididae from Persian Gulf.

The present paper has not been submitted to another journal, nor will it be in the 6 months after initial submission to *EJT*. All co-authors are aware of the present submission.

Abstract. The family Dendrodorididae O'Donoghue, 1924 is globally distributed in intertidal zones. In the Persian Gulf's northern coast three Dendrodorididae species were collected: *Dendrodoris fumata* (Rüppell & Leuckart, 1830), *Dendrodoris nigra* (W. Stimpson, 1855), and a new *Doriopsilla* species. Our study combines molecular, anatomical, and histological data to examine these species. Mitochondrial gene analyses, focusing on partial 16S rDNA and cytochrome oxidase I (COI) sequences, reveal a paraphyletic genus *Dendrodoris*, in contrast to results of nuclear gene studies. Two distinct groups within *Dendrodoris* species emerge separated by a long branch indicating introgression in one group. Our results confirm the differentiation between *Dendrodoris rubra* (Kelaart, 1858) and *D. fumata*. *Dendrodoris nigra*, *D. fumata* and *D. krusensternii* (Gray, 1850) consist of several clades, indicating cryptic species complexes requiring further investigation. We describe the presence of bacteria for the first time in the vestibular gland of *D. fumata*. Molecular analyses validate the identification of a new *Doriopsilla* species from the Persian Gulf, supported by haplotype networking, genetic distance, and ABGD analyses of mitochondrial genes. Finally, our study confirms a previous hypothesis that *Cariopsilla*, comprising the single species *Cariopsilla pharpa*, is a junior synonym of *Doriopsilla.*

Key words. Dendrodorididae; phylogeny; histology; species delimitation; haplotype networking

Introduction

Nudibranchia, known from all marine geographic areas, are usually found from the intertidal zone to the deep waters in different types of habitats (Klussmann-Kolb *et al*. 2008; Yonow 2015). Its highest diversity with approximately 2000 species is reported from tropical areas of the Indo-Pacific Ocean, but the diversity is assumed to be much higher (Gosliner *et al*. 2018). Some geographic areas experienced much more attention in the last years, mainly the species-rich Coral Triangle (Eisenbarth *et al*. 2018; Gosliner *et al*. 2018; Papu *et al*. 2022), while other areas are much less studied, e.g., the Iranian coast line. Most recently, Amini-Yekta & Dekker (2021) published a checklist on Gastropoda recorded from the Persian Gulf and the Gulf of Oman. Within this checklist 44 nudibranch species are mentioned of 850 gastropods in total. Thus, the number is considerably smaller than in other tropical areas, e.g., the Red Sea or the Coral Triangle (Yonow 2008; Papu *et al*. 2020). One reason for the low number of species lies possibly

in the harsh conditions of the Persian Gulf with higher average temperature and salinity, compared to the Indian Ocean. This is especially the case for the intertidal with extreme physio-chemical parameters (Maniei *et al*. 2020; Rezai *et al*. 2016). Only few nudibranch groups can be found regularly in the intertidal flats worldwide, and one of these is the family Dendrodorididae O'Donoghue, 1924. This family comprises three genera, *Dendrodoris* Ehrenberg 1831, *Doriopsilla* Bergh, 1880, and *Cariopsilla* Ortea & Espinosa, 2006. The genus *Dendrodoris* is listed with 44 species in the World Register of Marine Species (WoRMS) (http://www.marinespecies.org). Only two species are recorded from both the Persian Gulf and the Gulf of Oman: *Dendrodoris fumata* (Rüppell & Leuckart, 1830) and *Dendrodoris nigra* (W. Stimpson 1855) (Fatemi & Attaran 2015; Fatemi *et al*. 2021; Rezai *et al*. 2016; Mousavipoor 2013). *Dendrodoris krusensternii* (Gray, 1850) is listed in The Global Biodiversity Information Facility (GBIF; https://www.gbif.org) with one record from the Gulf of Oman. However, only two records of *D. nigra and D. fumata* are currently known from the Iranian coastline (Fatemi & Attaran 2015; Fatemi *et al*. 2021; Rezai *et al*. 2016; Mousavipoor 2013, master thesis in Persian language). The genus *Doriopsilla* has 26 species registered in WoRMS and is also represented in this region with two different species, *Doriopsilla nigrocera* Yonow, 2012 and *Doriopsilla* cf. *miniata*, both recorded once from the Persian Gulf only but not from the Iranian coastline (Yonow 2012). The only species assigned to the third genus within the Dendrodorididae, *Cariopsilla pharpa* (Er. Marcus, 1961) (originally described as *Doriopsilla pharpa*), is not found in this geographic area, but seems to be confined to the northwest Atlantic Ocean. The validity of this genus was doubted by Valdés & Hamann (2008), who considered it as a junior synonym of *Doriopsilla;* however, the genus is still listed as a valid taxon in the World Register of Marine Species (www.marinespecies.org/aphia.php?p=taxdetails&id=724106; last access 25th of August 2023)

Members of the Dendrodorididae do not possess a radula or jaws, a feature which the taxon shares with the five genera of the family Phyllidiidae and the monotypic Mandeliidae (Brodie 2001; Papu *et al*. 2022; Valdés & Gosliner 1999). These three taxa are nowadays united under the name Phyllidioidea. A close relationship was confirmed by morphological (Valdés & Gosliner 1999) and molecular studies (Furfaro *et al*. 2022; Papu *et al*. 2022; Thollesson 2020; Valdés 2003).

In recent years, species diversity and numbers has been increased especially by molecular studies, in which cryptic speciation and/or variation becomes much more obvious than in morphological studies. This also holds true for the Phyllidioidea. Stoffels *et al*. (2016) and especially Papu *et al*. (2022) demonstrated the underestimation of species numbers within the Phyllidiidae; Furfaro *et al*. (2022) revealed cryptic variation in *Doriopsilla areolata* Bergh, 1880. Interestingly, some of these studies were able to describe small morphological variations for the molecularly well-defined species. This is of interest especially in taxa, like *Dendrodoris*, which can exhibit considerable variations in external colours and patterns but have anatomical similarities, often causing taxonomic confusion, e.g., between *D. fumata* and *D. nigra* (Hirose *et al*. 2014). DNA sequencing and obtaining DNA barcodes enable species identification and delimitation despite these variations in colour patterns or other morphological features (Hirose *et al*. 2014). In the present study, we examined specimens of *D. nigra*, *D. fumata*, and a new *Doriopsilla* species found on the Iranian intertidal coastline of the Persian Gulf, using mainly histological methods. We analysed their partial CO1 and 16S rRNA gene sequences, including all available sequences of these genera and also using published sequences from the Persian Gulf (Fatemi *et al*. 2021). We applied various state of the art methods to clarify the species identities and their relationships within the genera.

Material and Methods

In total 12 specimens of *D. nigra*, 18 specimens of *D. fumata*, and 6 specimens of *Doriopsilla aroni* **sp. nov.** were collected from the southern coast of Iran (details summarised in Table 1). Sampling was undertaken in the intertidal zone during low tide at Bandar Lengeh (26°33′29″N 54°52′50″E), and Lavan Island (26°48′20.99″N 53°16′4.80″E) in February to April 2015 and March 2016 (Fig. 1A, C). Some animals were photographed alive using a digital camera (Canon SX160IS). The material is deposited in the Leibniz Institute for the Analysis of Biodiversity Change - Zoological Museum Hamburg (ZMH).

Table **1.** Information on the specimens and type material used for histology and/or DNA barcoding in this study.

Internal number/ museum number	Species	Locality and date of collection	Preservation/ Purpose of use	Length of preserved animal (mm)	GenBank accession COI	GenBank accession 16S
FM1 ZMH 141488	*Dendrodoris nigra*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	13	OR271872	OQ990331
FM2 ZMH 141488	*Dendrodoris nigra*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	10	OR271578	OQ990332
FM3 ZMH 141488	*Dendrodoris nigra*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	15	OR271686	OQ990340
FM4 ZMH 141488	*Dendrodoris nigra*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	13	OR282789	OQ990339
FM5 ZMH 141488	*Dendrodoris nigra*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	17	OR271929	OQ990338
FM6 ZMH 141488	*Dendrodoris nigra*	Bandar Lengeh 13.03.2015	96 % EtOH DNA barcoding	15	OR271968	-
FM7 ZMH 141488	*Dendrodoris nigra*	Bandar Lengeh 13.03.2015	96 % EtOH DNA barcoding	12	OR272035	OQ990337
FM8 ZMH 141488	*Dendrodoris nigra*	Bandar Lengeh 10.04.2015	96 % EtOH DNA barcoding	20	OR272037	OQ990336
FM9 ZMH 141488	*Dendrodoris nigra*	Bandar Lengeh 13.03.2015	96 % EtOH DNA barcoding	12	OR272038	OQ990335
FM10 ZMH	*Dendrodoris nigra*	Bandar Lengeh	96 % EtOH DNA barcoding	10	OR272039	OQ990334

141488		13.03.2015				
FM11 ZMH 141488	*Dendrodoris nigra*	Bandar Lengeh 10.04.2015	96 % EtOH DNA barcoding	24	OR282790	OQ990333
FM48	*Dendrodoris nigra*	Bandar Lengeh 13.03.2015	Formaldehyde/Seawater Histology	19	-	-
FM12 ZMH 141489	*Dendrodoris fumata*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	19	OR196687	OQ990325
FM13 ZMH 141489	*Dendrodoris fumata*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	17	OR208628	OQ990324
FM14 ZMH 141489	*Dendrodoris fumata*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	22	OR004588	OQ990323
FM15 ZMH 141489	*Dendrodoris fumata*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	15	OR213137	OQ990322
FM16 ZMH 141489	*Dendrodoris fumata*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	20	OR213144	OQ990321
FM17	*Dendrodoris fumata*	Bandar Lengeh 10.04.2015	96 % EtOH DNA barcoding	32	OR005512	OQ990320
FM18 ZMH 141489	*Dendrodoris fumata*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	30	OR044080	OQ990319
FM19 ZMH 141489	*Dendrodoris fumata*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	22	OR213187	OQ990318
FM20 ZMH 141489	*Dendrodoris fumata*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	20	OR213188	OQ990317

FM21 ZMH 141489	*Dendrodoris fumata*	Bandar Lengeh 05.03.2015	96 % EtOH DNA barcoding	17	OR213189	OQ990316
FM22 ZMH 141489	*Dendrodoris fumata*	Bandar Lengeh 10.04.2015	96 % EtOH DNA barcoding	28	OR213190	OQ990315
FM23 ZMH 141489	*Dendrodoris fumata*	Lavan Island 08.03.2016	96 % EtOH DNA barcoding	16	OR213191	OQ990314
FM24 ZMH 141489	*Dendrodoris fumata*	Lavan Island 08.03.2016	96 % EtOH DNA barcoding	15	OR213192	OQ990313
FM25 ZMH 141489	*Dendrodoris fumata*	Lavan Island 08.03.2016	96 % EtOH DNA barcoding	20	OR213193	-
FM26 ZMH 141489	*Dendrodoris fumata*	Lavan Island 08.03.2016	96 % EtOH DNA barcoding	26	OR213194	-
FM28 ZMH 141489	*Dendrodoris fumata*	Lavan Island 08.03.2016	96 % EtOH DNA barcoding	30	OR213195	-
FM29 ZMH 141489	*Dendrodoris fumata*	Lavan Island 08.03.2016	96 % EtOH DNA barcoding	24	OR213196	-
FM49	*Dendrodoris fumata*	Bandar Lengeh 08.03.2016	Formaldehyde/Seawater Histology	24	-	-
FM43 completely consumed	*Doriopsilla aroni* **sp. nov.**	Bandar Lengeh 14.02.2015	96 % EtOH DNA barcoding	24	OQ992500	OQ990330
FM44 ZMH 141486	*Doriopsilla aroni* **sp. nov.**	Bandar Lengeh 14.02.2015	96 % EtOH DNA barcoding **Paratype**	20	OQ992497	OQ990329

FM45 ZMH 141486	*Doriopsilla aroni* **sp. nov.**	Bandar Lengeh 14.02.2015	96 % EtOH DNA barcoding **Paratype**	24	OR018312	OQ990328
FM46 ZMH141485	*Doriopsilla aroni* **sp. nov.**	Bandar Lengeh 14.02.2015	96 % EtOH DNA barcoding **Holotype**	38	OQ992499	OQ990327
FM47 ZMH 141486	*Doriopsilla aroni* **sp. nov.**	Bandar Lengeh 14.02.2015	96 % EtOH DNA barcoding **Paratype** dissected	30	Q992498	OQ990326
FM50 ZMH 141487 histological slide series	*Doriopsilla aroni* **sp. nov.**	Bandar Lengeh 14.02.2015	Formaldehyde/Seawater **Paratype**	40	-	-

Fig. 1. Information on collection sites of Dendrodoridae specimens. **A.** Location of collection sites along Iranian coast line. **B.** Bandar Lengeh, collection site exposed at low tide. C. Lavan Island, collection site exposed at low tide.

Anatomical observation

For histological analyses, one specimen of *D. nigra* (FM48, Table 1), the genital system of one *D. fumata* (FM49, Table 1), and one specimen of *Doriopsilla aroni* **sp. nov**. (FM50, Table 1)

were embedded in hydroxyethyl methacrylate (Heraeus Kulzer GmbH) for serial sectioning. Sections (2.5 µm) were stained with toluidine blue and photographed subsequently under a ZEISS Microscope (Imager.Z2m). One specimen of *Doriopsilla aroni* **sp. nov.** (FM47, Table 1) was dissected under a stereomicroscope (Wild M8 ZOOM).

DNA extraction, PCR, and DNA sequencing

In total, we sequenced 11 *Dendrodoris nigra*, 17 *D. fumata* and 5 *Doriopsilla aroni* **sp. nov.** specimens. To retrieve the CO1 and 16S sequences from our new collected material, we used the primer designed by Astrin & Stüben (2008) and Palumbi *et al*. (1991), and the settings of the amplification, trimming and quality control as described in Papu *et al*. (2022). Sequences are deposited in GenBank with the accession numbers listed in Table 1.

Phylogenetic reconstruction

All available CO1 and 16S sequences of the family Dendrodorididae (248 sequences) were downloaded from GenBank (for more details see Table S1) and added to our alignment. Five species of the family Phyllidiidae and further dorids were included to better understand the relationship of the genera within Phyllidioidea. Two members of the Bathydorididae were used to root the trees (Table S1). Additionally, all available nuclear sequences of the 18S and H3 genes of *Doriopsilla*, *Dendrodoris* and phyllidiid species, as well as several dorid specimens, with two sequences of Bathydorididae as outgroup were downloaded from GenBank (Table S1) and analysed separately from the two mitochondrial gene data sets (Table 2).

Sequences were edited using BioEdit (ver.7.2.6.1) (Hall 1999) and aligned using MAFFT (Katoh *et al*. 2002) in Geneious version 7.1.9 (Kearse *et al*. 2012). After trimming, the alignments of the mitochondrial genes comprised 501 bp for 16S, 640 bp for CO1 and 1141 bp for the concatenated dataset. The alignments of the nuclear genes comprised 2082 bp for 18S and 345 bp for H3. In-depth genetic analyses were partially performed on reduced datasets by confining the complete CO1 and 16S datasets to only *Dendrodoris* or *Doriopsilla* sequences and outgroups. We refer here to Table 2 for details of these alignments.

Maximum likelihood (ML) analyses were run in IQ-TREE (Nguyen *et al*. 2014; Trifinopoulos *et al*. 2016) using the online version 1.6.3 on a webserver (http://iqtree.cibiv.univie.ac.at/), with the GTR model for all genes and gene data sets (see Table 2). Support values were calculated based

on 1000 ultrafast bootstrap replicates and the approximate likelihood ratio test (SH-aLRT) (2000 replicates). Dendroscope (version 3.5.8) (Huson & Scornavacca 2017) and Inkscape (version 0.92) (https://inkscape.org/en/) were used to edit the phylograms.

Species delimitation and genetic distances

For species delimitation, we analysed CO1, 16S, 18S, and H3 datasets and the *Doriopsilla* subset applying the Automatic Barcode Gap Discovery (ABGD) methodology (Puillandre *et al.* 2011) (Table 2), the same program that was used recently for investigation of *Dendrodoris* species (Galià-Camps *et al.* 2022). We used the default settings under the Kimura K80 model. The minimum and maximum pairwise uncorrected p-distances of the reduced 16S and CO1 subsets between and within species or main clades were also calculated with this programme (Table 2).

Haplotype networks of *Doriopsilla*

A statistical parsimony analysis (Templeton *et al*. 1992) was performed on the same reduced *Doriopsilla* subset using the program TCSv.1.21 (Clement *et al*. 2000) in PopART (Liegh & Bryant. 2015) (Table 2). Settings used were a 95% connection limit and 5,000 iterations. In the haplotype analysis, we also included and visualized geographic information available for each specimen.

Table 2. Summary of molecular datasets used in this study and performed analyses with indication of respective tables and figures in text and supplement.

	Number of sequences (Dendrodorididae /other dorids/ outgroups)	Phylogeny	ABGD Test	Genetic distance analysis	Haplotype analysis
Concatenated 16S and CO1 complete dateset	247/19/2 Bathydorids	Fig. 10	No	No	No
CO1 complete dateset with all Dendrodorididae	202/19/2 Bathydorids	Fig. 12	Fig. 12	No	No
16S complete dataset with all Dendrodorididae	198/19/2 Bathydorids	Fig. 11	Fig. 11	No	No
18S Dendrodorididae	17/10/2 Bathydorids	Fig. S1	Fig. S1	No	No
H3 Dendrodorididae	115/10/2 Bathydorids	Fig. S2	Fig. S2	No	No
16S subset only *Dendrodoris*	71/0/-	No	Yes	Table S2	No
CO1 subset only *Dendrodoris*	132/0/-	No	Yes	Table S3	No
16S subset only *Doriopsilla*	98/0/3 *Dendrodoris*	Fig. S3	Fig. S3	Table 3	Fig. 14
CO1 subset only *Doriopsilla*	76/0/4 *Dendrodoris*	Fig. S4	Fig. S4	Table 4	Fig. 13

Results

Taxonomic descriptions

Class Gastropoda Cuvier, 1795

Order Nudibranchia Cuvier, 1817

Family Dendrodorididae O'Donoghue, 1924 (1864)

Genus *Dendrodoris* Ehrenberg, 1831

Type species *Dendrodoris limbata* Cuvier, (1804) (type by subsequent designation)

***Dendrodoris nigra* (W. Stimpson, 1855)**

Figs 2A, B, 3, 4, 5A

A comprehensive list of synonyms is provided by Brodie *et al.* (1997).

Diagnosis

Body soft, without spicules, typically, the coloration black or dark grey. Delicate mantle margin. More than 5 gill branches. Oral tentacles absent. Ptyalin glands and salivary glands present; oesophagus characterised by a highly glandular epithelium. Penis with spines.Vestibular gland present in reproductive system (Brodie *et al*. 1997; Valdés *et al*. 1996; Valdés & Gosliner 1999; Wägele *et al*. 1999; Yonow 2012).

Material examined

IRAN • 12. Bandar Lengeh; 26°33′29″N 54°52′50″E; Mar.-Apr. 2015; Fatemeh Maniei leg.; LIB Zoological Museum Hamburg; ZMH 141488

Description

Body length of the 12 preserved animals: 10-24 mm

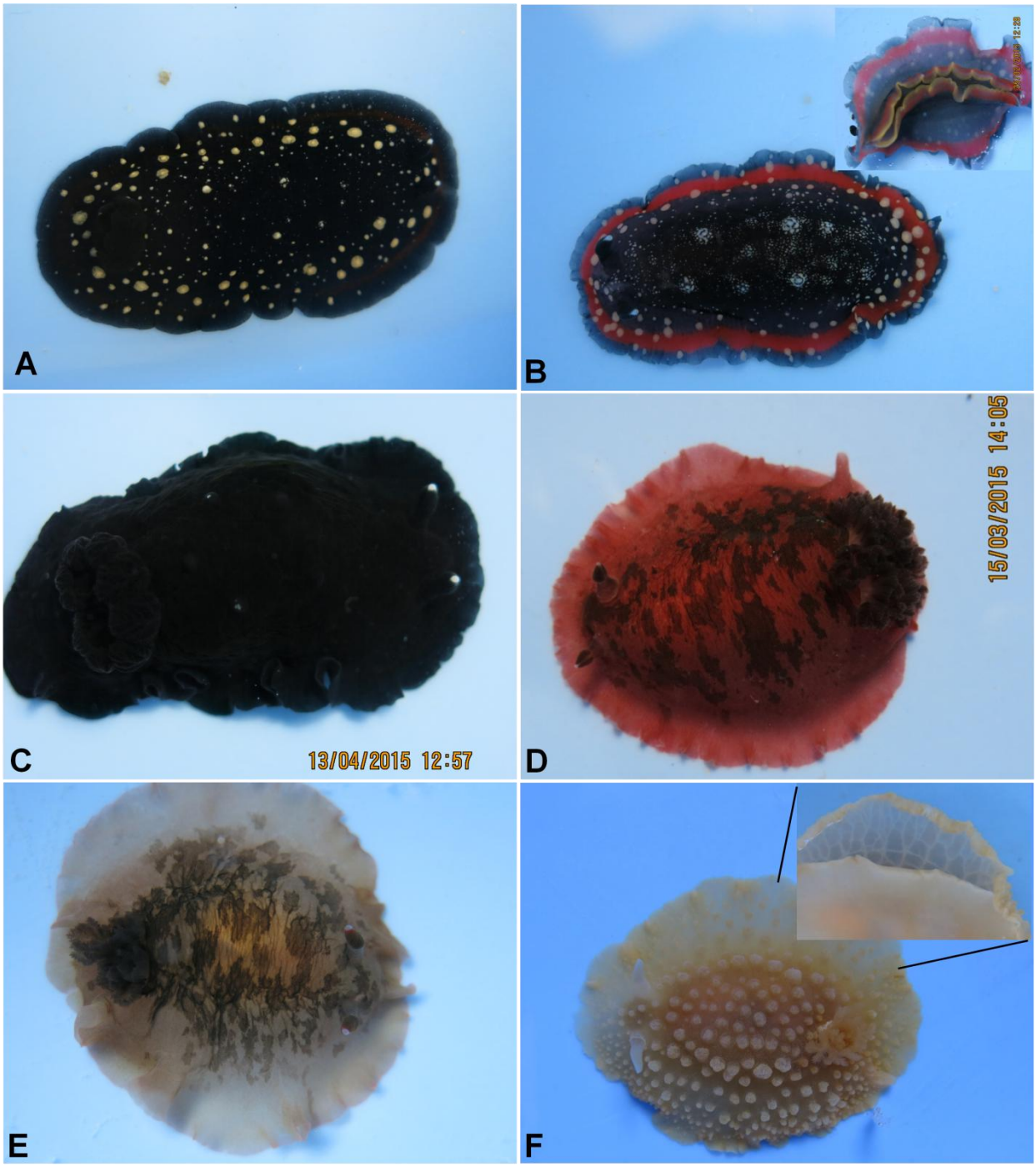


Fig. 2. External appearance of live animals. A. Dorsal view of *Dendrodoris nigra* (W. Stimpson, 1855) with black mantle and yellow spots. B. *Dendrodoris nigra* with black mantle, white spots, and a thick distinct red submarginal band; inset shows ventral view. C. *Dendrodoris fumata* (Rüppell & Leuckart, 1830) black form. D. *Dendrodoris fumata* red form. E. *Dendrodoris fumata* pale brown form. F. Dorsal view of *Doriopsilla aroni* **sp. nov.**; inset shows ventral view.

Morphology

Live animals elongate with a soft body, the almost smooth mantle forming a wavy edge. Two colour variants observed: black mantle, with many white to yellow spots and with a pale red band around the mantle (Fig. 2A), or black mantle with white spots and a thick distinct red submarginal band around the notum margin and around foot margin, bordered by an outer yellow band (Fig. 2B). The rhinophores always black with a white apex and about 12 lamellae on each rhinophore clavus. Six pinnate gills present, arranged in a circle around the anus and curved inwards in life.

Histology

One black specimen with a distinct red submarginal band (length of preserved animal 19 mm) was examined histologically. The slide series is deposited in the histology collection of the LIB, Museum Koenig Bonn, without a number.

Integument

Epidermis of the mantle of cuboidal cells with (mucus cells interspersed; mucus cells with homogeneously stained purple (acid mucopolysaccharides) or with dark purple grana. (Fig. 3A). Epithelium of hyponotum with cuboidal or flat cells and fewer glandular cells. Melanin grana lying subepidermal, distributed in patches. Epithelium of the foot with high columnar cells and subepidermal mucous glands with uniformly pale purple contents.

Digestive system

Mouth area surrounded by a thick violet stained glandular layer containing high columnar cells. Oral tube with cuboidal to columnar cells, interspersed with reddish stained glandular cells (Fig. 3B). A paired ptyalin gland present with separate ducts leading into one central muscular duct finally opening dorsally into the oral tube, close to the transition into the pharynx; cells of ptyalin gland with pyknotic nuclei and no visible contents, thus creating a spongy appearance. Ducts inside the gland composed of glandular cells with pale bluish stained grana. Labial disc with reddish staining subepithelial glandular cells, and without cuticle (Fig. 3B). The pharynx with a triangular-shaped lumen. Epithelium consisting of cuboidal to elongate cells, covered at least in the proximal part of pharynx by a very thin cuticle. Glandular cells absent. Two muscular layers

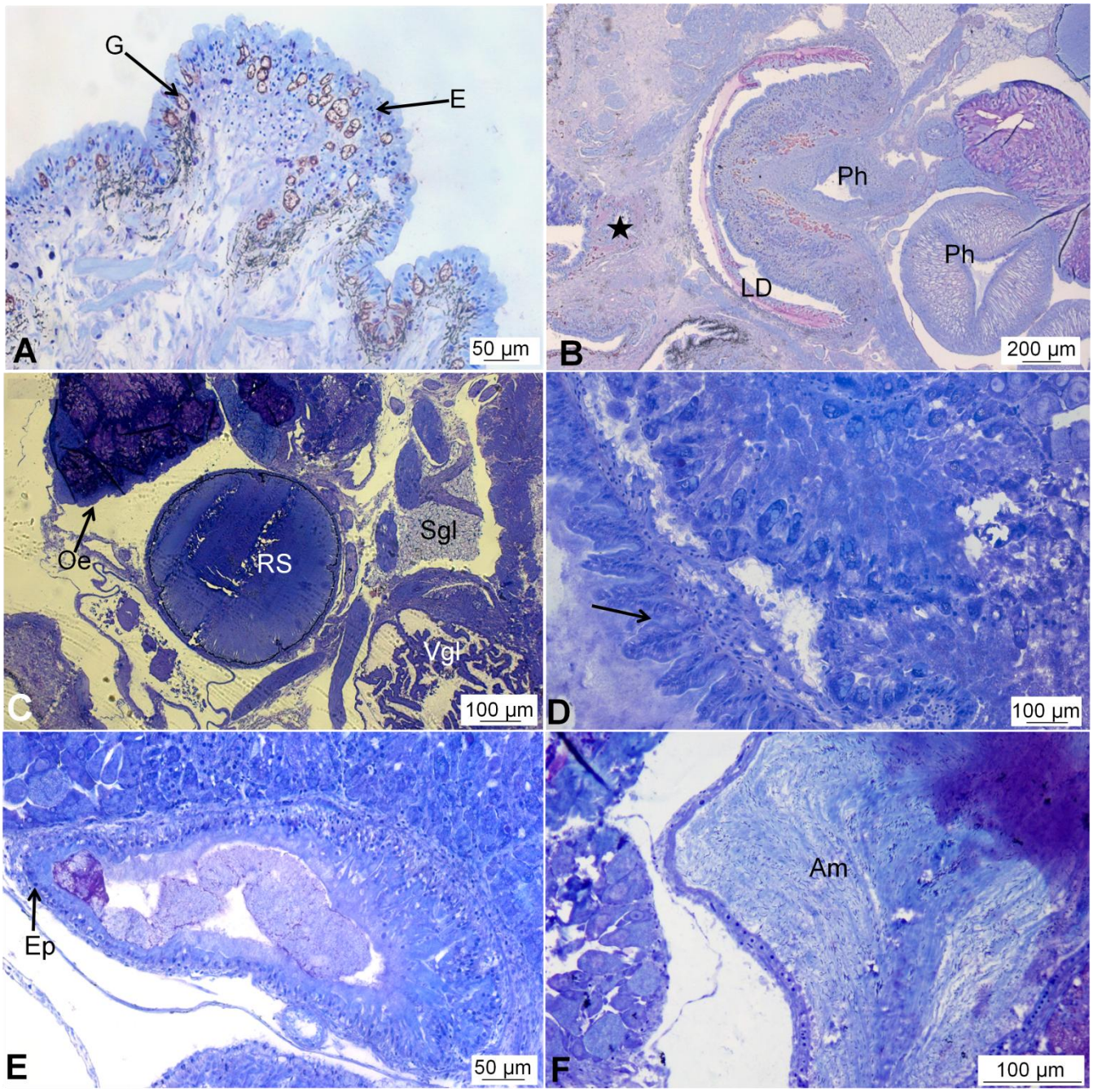


Fig. 3. Histological cross sections of epidermis, digestive system, and part of the genital system of *Dendrodoris nigra* (W. Stimpson, 1855). **A**. Mantle. **B**. Head area with parts of the digestive system; the oral tube is marked with a star. **C**. Section behind the head region with parts of the genital system. **D**. Stomach with folded wall (arrow). **E**. Intestine. **F**. Ampulla. Abbreviations: Am = Ampulla; E = Empty unicellular gland; Ep = Epithelium; G = glandular vesicle with mucopolysaccharides (stained violet); LD = Labial disc; Oe = Oesophagus; Ph = Pharynx; Sgl = Salivary gland; Vgl = Vestibular gland; Rs = Receptaculum seminis.

surrounding the pharyngeal epithelium; the inner one with fibres oriented transversely, which enlarge the lumen when contracted; the outer layer with fibres arranged circularly, which serve to reduce the lumen of the pharynx, and lengthen it (Fig. 3B). Salivary glands small, with small spherical cells filled with pale bluish stained grana and large nuclei (Fig. 3C). Oesophagus thicker in the cross section than the pharynx. Epithelium of the oesophagus highly folded, consisting of high columnar glandular cells staining light to dark violet (Fig. 3C). In some areas these cells with granular, in others with homogeneously stained contents. Ciliated cells interspersed. Stomach sac-like and fully embedded within digestive gland, with folded epithelium; cells elongate and ciliate (Fig. 3D). Digestive gland lobulated or folded on dorsal side, with gonad and kidney lying in these folds. Intestine arising from the embedded stomach; its folded epithelium surrounded by an inner circular and outer longitudinal muscle layer. Epithelial cells of the intestine elongate, ciliated, and non-glandular (Fig. 3E). No caecum and typhlosole observed.

Reproductive system

Gonad forming thick layer on dorsal side, composed of separate areas of oocyte and spermatocyte production. Sausage-shaped ampulla filled with autosperm. Epithelium of ampulla formed by cuboidal cells (Fig. 3F). Prostatic part of vas deferens with subepithelial glandular layer, gland cells with pale bluish stained grana (Fig. 4A). Penis very long, without cuticle, but with spines. Receptaculum seminis surrounded by muscle layer; sperm heads oriented or attached to the rather flat epithelial cells (Fig. 3C). Bursa copulatrix filled with degrading sperm and probably prostatic material and eggs; epithelium partly composed of apocrine secernating cells. Bursa connecting to short distal vaginal duct, the latter opening in a common vestibulum with vas deferens. Nidamental glands already large despite the small size of the animal, consisting of three areas, staining in different shades from red to bluish and dark violet. Vestibular gland large, partly lying in the body cavity, partly in the notum tissue (Fig. 3C); connecting to distal oviduct. Its heavily folded epithelium consisting of small, dark blue stained cells with a thick layer of microvilli in which symbiotic bacteria appear to reside (Fig. 4B). A general outline of the reproductive system is given in Fig. 5A.

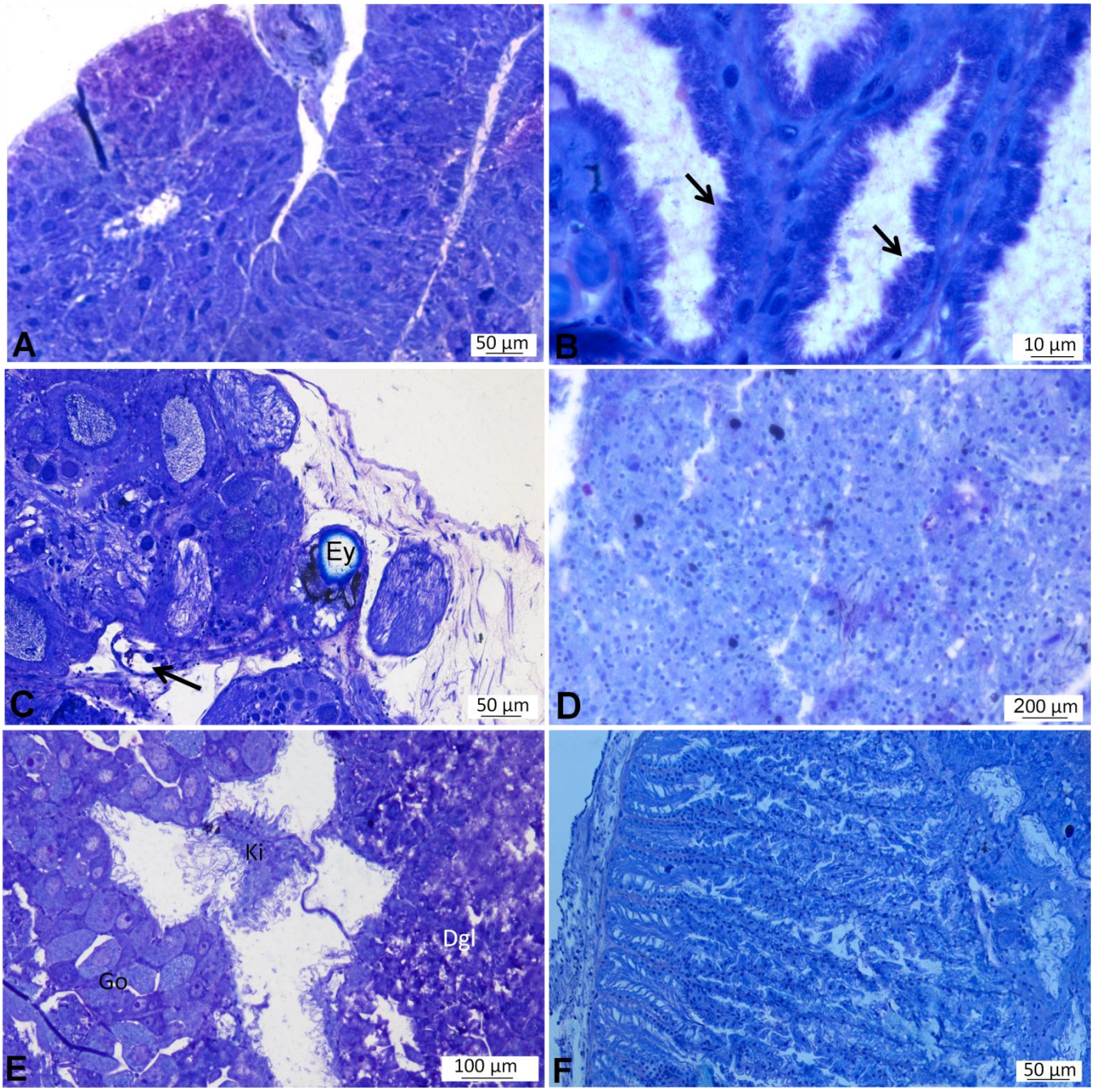


Fig. 4. Histological cross sections of sensory organs and genital, circulatory, and excretory systems of *Dendrodoris nigra* (W. Stimpson, 1855). A. Prostate gland. B. Vestibular gland with high microvilli edge (arrows). C. Statocyst with several otoconia (arrow) and eye. D. Blood gland. E. Kidney, digestive gland, and gonad. F. Syrinx; note the highly folded interior. Abbreviations: Go = Gonad; Ki = Kidney; Dgl = Digestive gland.

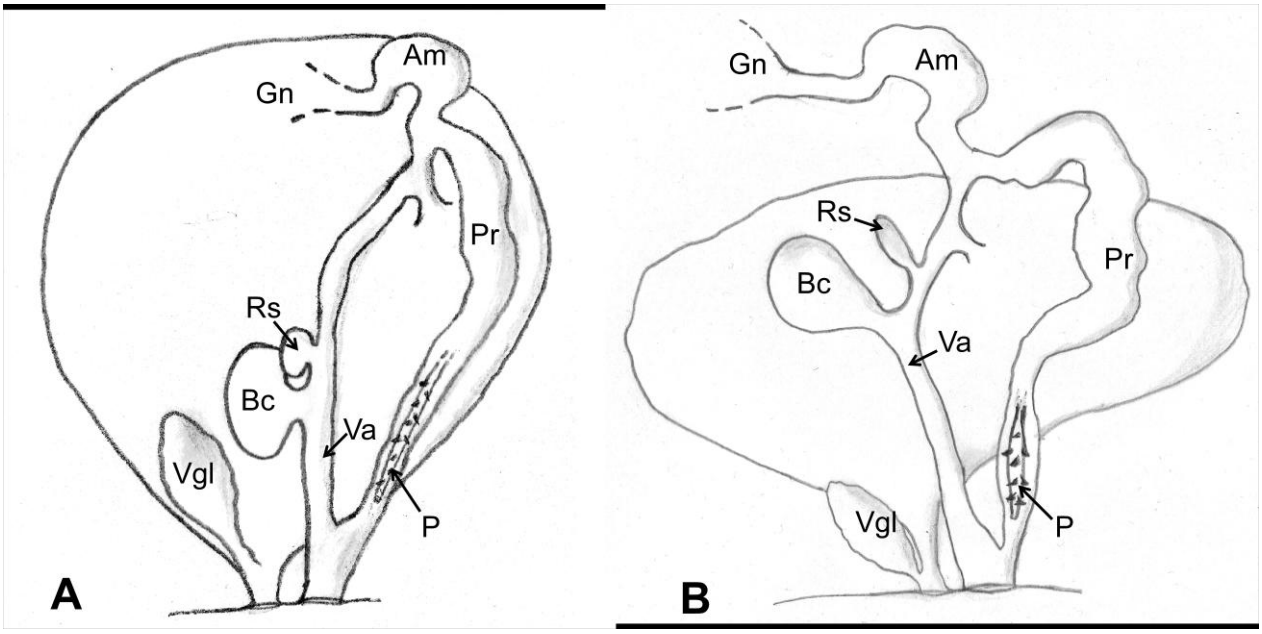


Fig. 5. Schematic outline of genital system of *Dendrodoris* Ehrenberg, 1831 species. **A.** *D. nigra* (W. Stimpson 1855). **B**. *D. fumata* (Rüppell & Leuckart, 1830). Abbreviations: Am = Ampulla; Bc = Bursa copulatrix; Gn = Gonad; P = Penis; Pr = Prostate gland; Rs = Receptaculum seminis; Va = Vaginal duct; Vgl =Vestibular gland.

Nervous system

Cerebropleural complex at the transition of pharynx into the oesophagus. Statocyst with many otoconia (Fig. 4C).

Circulatory system

Ventricle very muscular. Blood gland close to cerebropleural complex, containing small, dark blue, stained cells (Fig. 4D).

Excretory system

Kidney lying intermingled between gonad and digestive gland; with large cuboidal cells with no stained contents (Fig. 4E). Syrinx very large, forming bulb-like structure with highly folded epithelium and long cilia (Fig. 4F).

Additional features

Body cavity surrounded by a very thick muscle layer, especially in the hind part of the body. A large retractor muscle originating close to the gills, dividing into two main muscles leading ventrally towards the anterior part, finally fusing with a strong ventral muscle layer in the anterior body. Small gill glands present at the base of the gills, opening into the gill pocket; glandular cells with pale bluish cytoplasm and large active nuclei.

***Dendrodoris fumata* (Rüppell & Leuckart, 1830)**

Figs 2C–E, 5B, 6

A comprehensive list of synonyms is provided by Brodie *et al.* (1997).

Diagnosis

Body soft, more oval when crawling and with a more wavy mantle margin than in *D. nigra*. Body colour light brown to translucent red/orange to almost black; specimens with lighter colours often with irregular dark patches on notum. Five or six large gills. Penis with spines. In the present study we confirm the presence of a vestibular gland.

Material examined

IRAN • 18; Bandar Lengeh; 26°33′29″N 54°52′50″E; Mar-Apr. 2015 and Lavan Island; 26°48′20.99″N 53°16′4.80″E; Mar. 2016; Fatemeh Maniei leg.; LIB Zoological Museum Hamburg; ZMH 141489.

Description

Body length of preserved animals: 15-32 mm

Morphology

Body of investigated live animals elongate, with soft and smooth notum. Margin of the notum wavy, thin, and wide. Body colour variable: completely black with traces of a submarginal red-orange band around the anterior end of the mantle (Fig. 2C), red (Fig. 2D) or pale brown (Fig. 2E). The pale brown animals with a large number of dark brown patches on the dorsal surface,

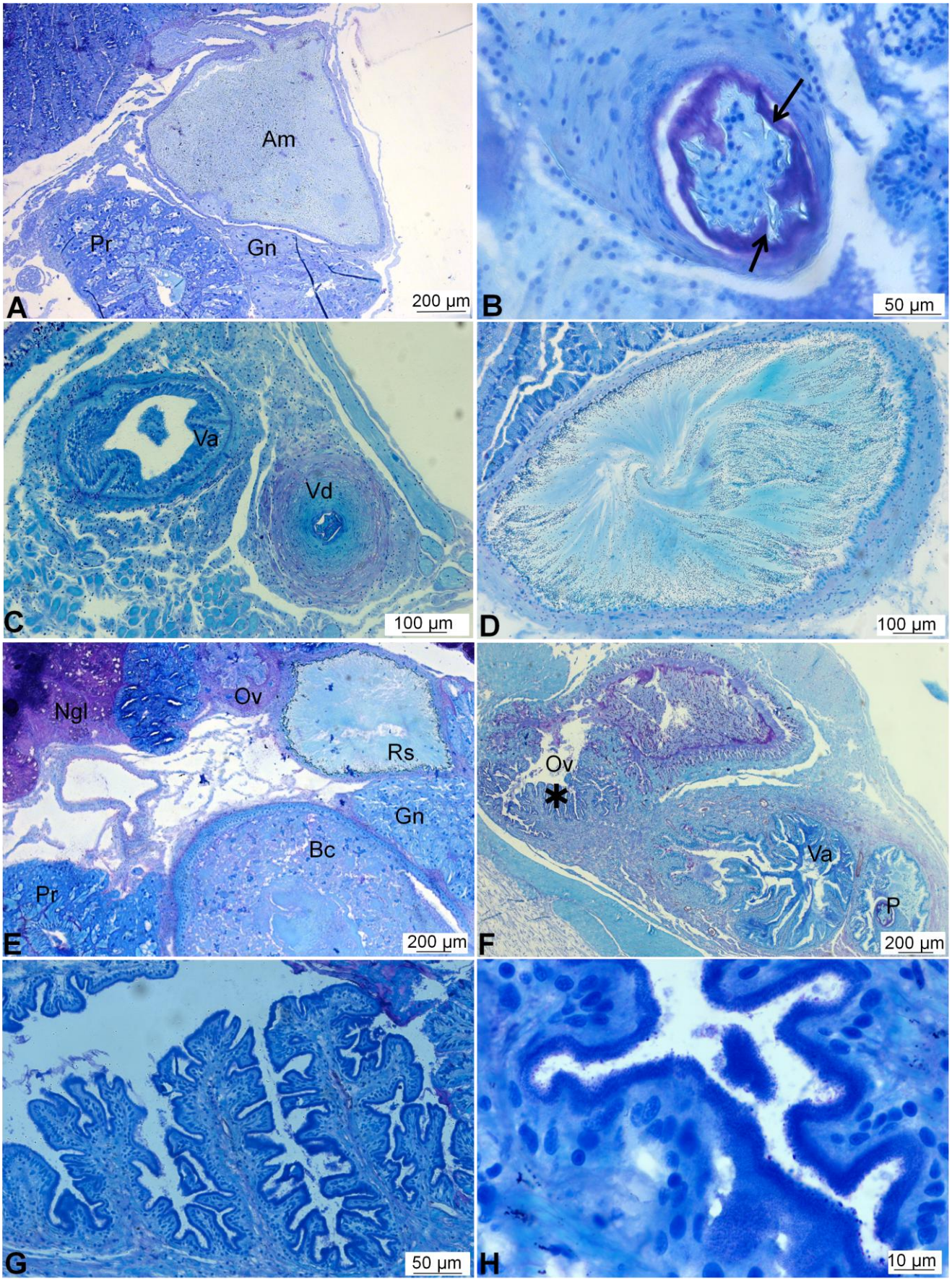
except at the margin. Rhinophores of the same colour as the dorsal surface, but always with a white apex. Clavus with 10-16 lamellae in larger animals. Five large branched gills, usually somewhat darker than dorsal notum.

Histology

Reproductive system of one specimen (red form, 24 mm in length, collected in Bandar Lengeh 2015) was extracted and investigated separately by histological means. The slide series is deposited in the histology collection of the LIB, Museum Koenig Bonn (without number).

Reproductive system

Gonad intermingled with the digestive gland and kidney. Ampulla filled with autosperm; epithelium thin with cuboidal cells (Fig. 6A). Non-prostatic part of vas deferens with elongate and ciliated epithelial cells. The prostate gland partly accompanying vas deferens, composed of epithelium with large glandular cells, staining pale blue; thick layer of subepithelial glandular cells present with small, dark blue, stained cells (Fig. 6A). Penis equipped with spines and thick cuticular layer, ending inverted in long penial sheath (Fig. 6B). Vagina and vas deferens opening into vestibulum. Vagina with thick folded walls containing subepithelial glandular cells (Fig. 6C). Receptaculum seminis with epithelium composed of small cuboidal cells, and surrounded by thick muscle layer (Fig. 6D); with sperm partly attached to the wall and partly lying in the lumen. Bursa copulatrix with thin epithelium composed of cuboidal cells, without underlying muscle layer (Fig. 6E). Female gland with three distinguishable parts: capsule, membrane, and mucous gland staining from red to dark violet (Fig. 6E). Large vestibular gland attached to distal oviduct; partly lying in notal tissue and partly in body cavity; internally highly folded, whereas oviduct less folded in this area (Fig. 6F). Epithelium of vestibular gland with small elongate cells, but thick microvillous border, in which bacteria are assumed to reside (Fig. 6G-H). Epithelium of distal oviduct in this area with small, elongate, ciliated cells. Small epithelial gland cells interspersed, staining pale violet. These are not present in the oviduct part with presumed bacteria. Distal oviduct terminally tube-like, with small mucus cells, and opening outwards next to vestibulum. A nematode and another unidentified parasite were observed in the vicinity of the genital opening (Fig. 6F).


Am
Pr
Gn
200 µm
A
50 µm
B
Va
Vd
100 µm
C
100 µm
D
Ngl
Ov
Rs
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Bc
Pr
200 µm
E
Ov
Va
P
200 µm
F
50 µm
G
10 µm
H

Fig. 6. Histological cross sections of genital system of *Dendrodoris fumata* (Rüppell & Leuckart, 1830). **A**. Gonad, ampulla, and prostate. **B**. Penis with spines (arrowed) inside the muscular sheath. **C**. Vaginal duct and vas deferens. **D**. Receptaculum seminis. **E**. Gonad, prostate, bursa copulatrix, receptaculum seminis, proximal oviduct and a part of nidamental gland. **F**. Strongly folded vestibular gland starting from less folded oviduct. **G.** Vestibular gland. **H.** Vestibular gland. Note the thick microvilli fringe. Abbreviations: Am = Ampulla; Bc = Bursa copulatrix; Gn = Gonad; Ngl = Nidamental gland; Ov = Oviduct; P = Penis; Pr = Prostate gland; Rs = Receptaculum seminis; Va = Vaginal duct; Vd = Vas deferens.

Genus *Doriopsilla* Bergh, 1880

Type species *Doriopsilla areolata* Bergh, 1880 (type species by monotype)

***Doriopsilla aroni* Maniei & Wägele, sp. nov.**

(Figs 2F, 7-9)

Diagnosis

Size up to 40 mm or slightly longer, white pigmentation or lines on the notum or rhinophores absent, except for a tiny line along the rim of the gill pocket. Dorsal notum covered by tubercles, in which many spicules intrude. Oral tentacles fused. Five gill plumes present. Anus eccentric. Pyloric gland present. Prostate gland forming a large mass. Penial hooks arranged in longitudinal lines in the proximal part of eversible penis. Spicules forming a thick layer around visceral cavity. Small gill glands present.

Etymology

Doriopsilla aroni **sp. nov.** is named after the first author's young son, Aron.

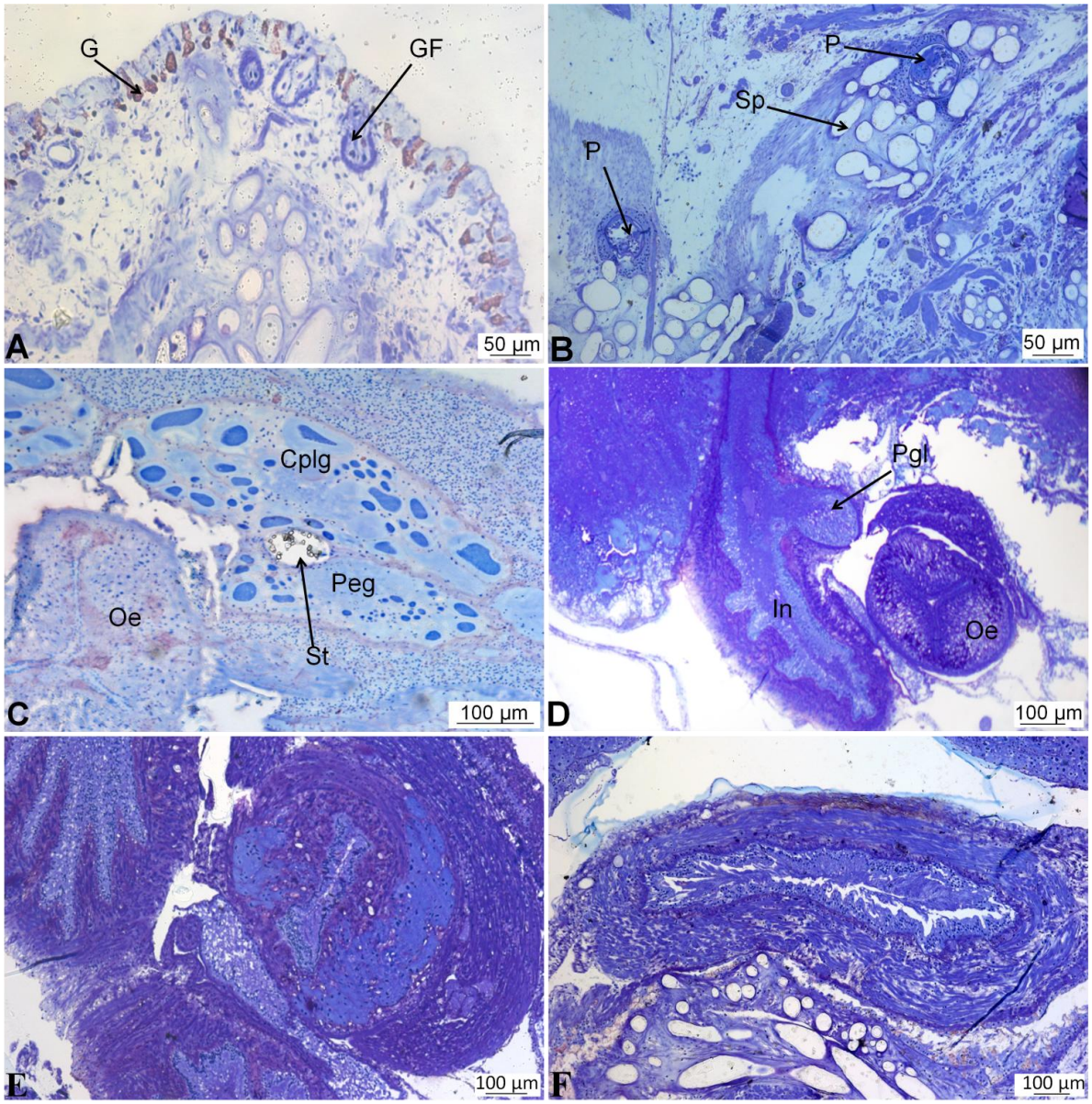

Fig. 7. Histological sections of *Doriopsilla aroni* **sp. nov.**. A. Cross section of tubercles containing spicules. B. Cross section of the mantle with spicules; two parasites can be seen in the notum tissue. C. Cross section of oesophagus and part of cerebropleural/pedal ganglia with statocyst. D. Cross section of oesophagus and opening of intestine into stomach with pyloric gland. E. Cross section of muscular oesophagus. F. Cross section of highly ciliated and muscular intestine. Abbreviations: Cplg = Cerebropleural ganglion; G = Glandular vesicle containing mucopolysaccharides (stained in violet); GF = Glandular follicles surrounded by muscle layers; In = Intestine; Oe = Oesophagus; P = Parasite; Peg = Pedal ganglion; Pgl = Pyloric gland; Sp = Spicules; St = Statocyst with otoconia.

Material examined

Holotype

IRAN • Bandar Lengeh; 26°33′29″N 54°52′50″E; 14 Feb. 2015; Fatemeh Maniei leg.; LIB Zoological Museum Hamburg; ZMH 141485.

Paratypes

IRAN • 4 same collection data as for holotype; ZMH 141486 3 spcms, ZMH 141487 histological slide series.

Description

Body length of preserved animals: holotype 38 mm, paratypes 20-40 mm.

Morphology

Colour of living animals varying from cream yellow to deep orange or vermilion. No white pigmentation on notum or rhinophores (Fig. 2F), except for a thin white line around the rim of the gill pocket. Body oval. Notum covered with rounded tubercles of approximately the same size, becoming smaller only along mantle margin; their colour sometimes a little bit paler than the notum. Hyponotum nearly translucent or cream in colour, stiffened by a network of spicules (Fig. 2F). Cylindrical rhinophores with 11-13 lamellae, same colour as the tubercles but with an apex brighter in colour. Five gill plumes (two pointing forward and three backward) located in a gill pocket. Anus eccentric to the left side of the gill. Oral tentacles small and fused. Fleshy muscular foot rounded anteriorly and more acute posteriorly.

Histology

The preserved specimen (ZMH 141487) used for histological examination had a length of 40 mm. The slide series is deposited in the collection of the LIB, Zoological Museum Hamburg (ZMH 141487).

Integument

Dorsal notum epithelium with many single subepithelial glandular cells staining red to violet and glandular follicles composed of several large bluish gland cells. These glandular follicles surrounded by muscle layers (Fig.7A). Spicules mainly present in the tubercles, around the visceral cavity (Fig. 7A, B), and in the foot.

Digestive system

A schematic outline of the digestive tract is given in Fig. 8A.

Mouth opening non glandular. Proximal oral tube highly folded with few epithelial mucus glands staining reddish. Subsequent part widening, with increasing number of epithelial acid mucus cells. Distal part of oral tube widening considerably, surrounded by muscle fibres and dorsally with subepithelial glands staining violet. Gradual transition into pharynx. Pharynx oval, surrounded by muscle fibres and with a slightly folded, glandular epithelium. Subepithelial glands reaching into muscle layer. No cuticle observed. Transition of pharynx into oesophagus marked by the location of the ganglionic nervous system (Fig. 7C). At this transition, two lateral retractor muscles attached. Salivary glands absent. Tubular oesophagus with Y-shaped interior lumen and no glandular epithelium (Fig. 7D), subsequently entering a highly muscular portion without Y-shaped lumen (Fig. E) and with few subepithelial glands, staining pale blue. Two small retractor muscles arising from this muscular part. After the muscular part oesophagus transiting into a small tube before entering the stomach. Stomach completely embedded in digestive gland; characterised by folded and ciliated epithelium. Transition into intestine with folds, but without a distinct typhlosole (Fig.7D-E). Pyloric gland opening immediately after transition of stomach into intestine; internally folded but without glandular cells, and filled with stomach contents (Fig. 7D). Interior lining of the intestine highly folded and ciliated, especially in the anal papilla. Intestine surrounded by a muscular layer throughout its course (Fig. 7F). Besides the oral gland layer, no ptyalin or salivary glands present. No caecum found.

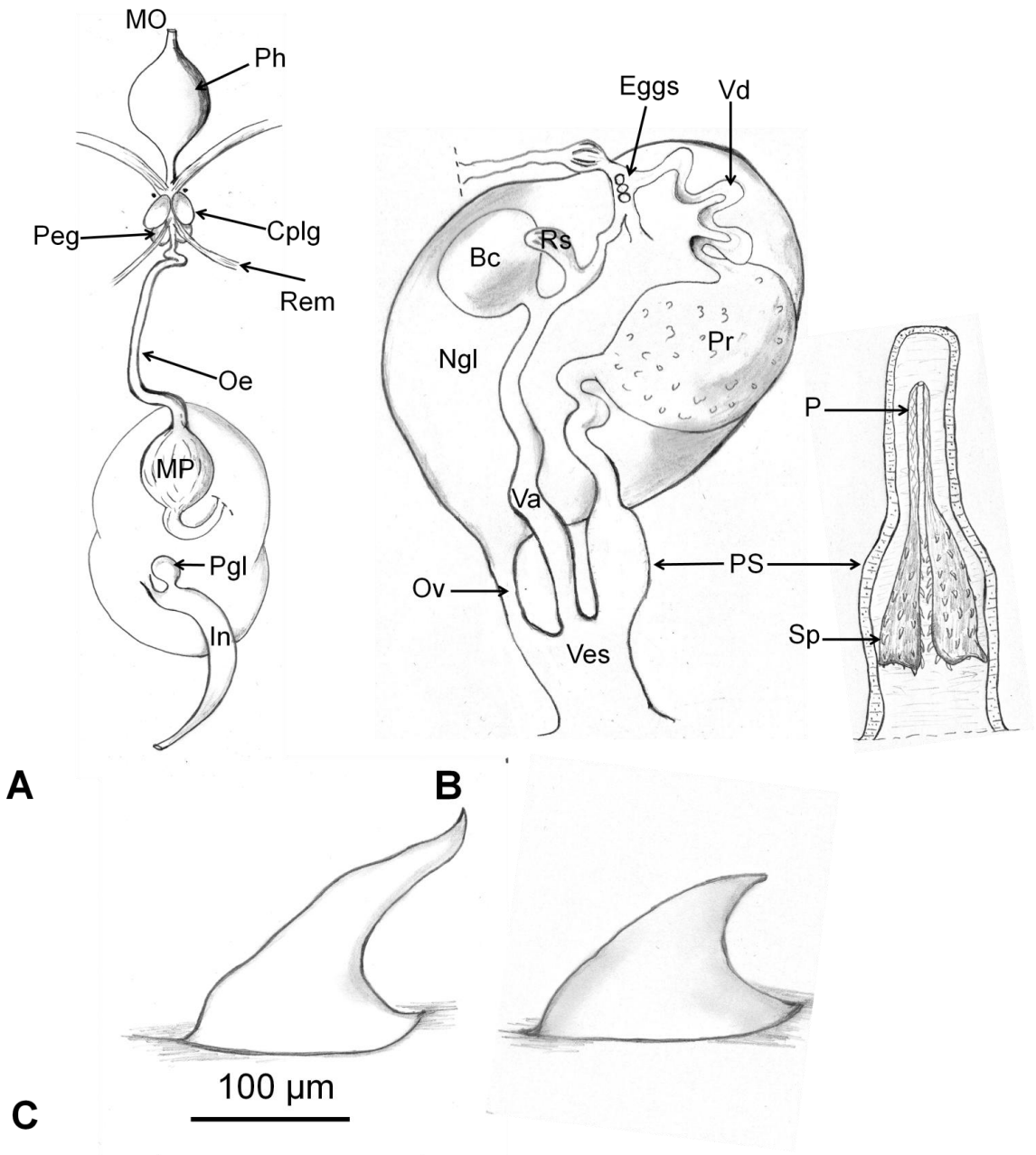

Fig. 8. Schematic outline of digestive and genital system of *Doriopsilla aroni* **sp. nov.**. A. Digestive system. B. Genital system and separate schematic outline of inverted penis. C. Penial spines of different shapes. Abbreviations: Bc = Bursa copulatrix; Cplg = Cerebropleural ganglion; In = Intestine; MO = Mouth opening; MP = Muscular portion of oesophagus; Ngl = Nidamental gland; Oe = Oesophagus; Ov = Oviduct; P = Penis; Peg = Pedal ganglion; Ph = Pharynx; Pgl = Pyloric gland; Pr = Prostate gland; Rem = Retractor muscle; Rs = Receptaculum seminis; Va = Vagina; Vd = Vas deferens; Ves = Vestibulum.

Reproductive system

A schematic outline of the genital system is provided in Fig. 8B. Gonad in mature phase with sperm and eggs formed in the same follicles. A distinct ampulla not found. Vas deferens leading into strongly lobulated, bulky prostate gland (Fig. 9A), showing pristine glandular areas, with small cells and large nuclei, other areas with elongate cells and vesicles with blue stained droplets, as well as some areas where the prostatic material was already exuded. Distal vas deferens (ejaculatory duct) coiled, lying in muscular sheath (Fig. 9B); penis long, inverted, with hooked spines only in the proximal first part, which lies in the wider part of the penial sheath. Distal second part without spines (Figs 8B-C, 9C). According to one dissected paratype (ZMH141486) spines arranged in longitudinal lines along the proximal penis. Size of spines around 200 µm, some of them smaller and thinner in the area, where spines start. Bursa copulatrix stalked and filled with decaying sperm and prostatic material; epithelium thin and characterised by apocrine secreting cells (Fig. 9E). Bursa copulatrix connected to receptaculum seminis by a muscular duct. Muscular receptaculum seminis empty, indicating a recent fertilisation process (Fig. 9F). Fertilisation chamber with a few eggs (Fig. 9D). Female glands exhibiting several areas with high columnar cells staining violet or reddish, sharing a joint non glandular vestibulum with vas deferens and the vaginal duct.

Nervous system

Statocyst between cerebropleural and pedal ganglia, with many otoconia (Fig. 7C).

Circulatory and excretory system

Blood gland surrounding the central nervous system and also the anterior part of the oesophagus. Syrinx highly folded and rather small. Kidney thin, covering gonad and digestive gland.

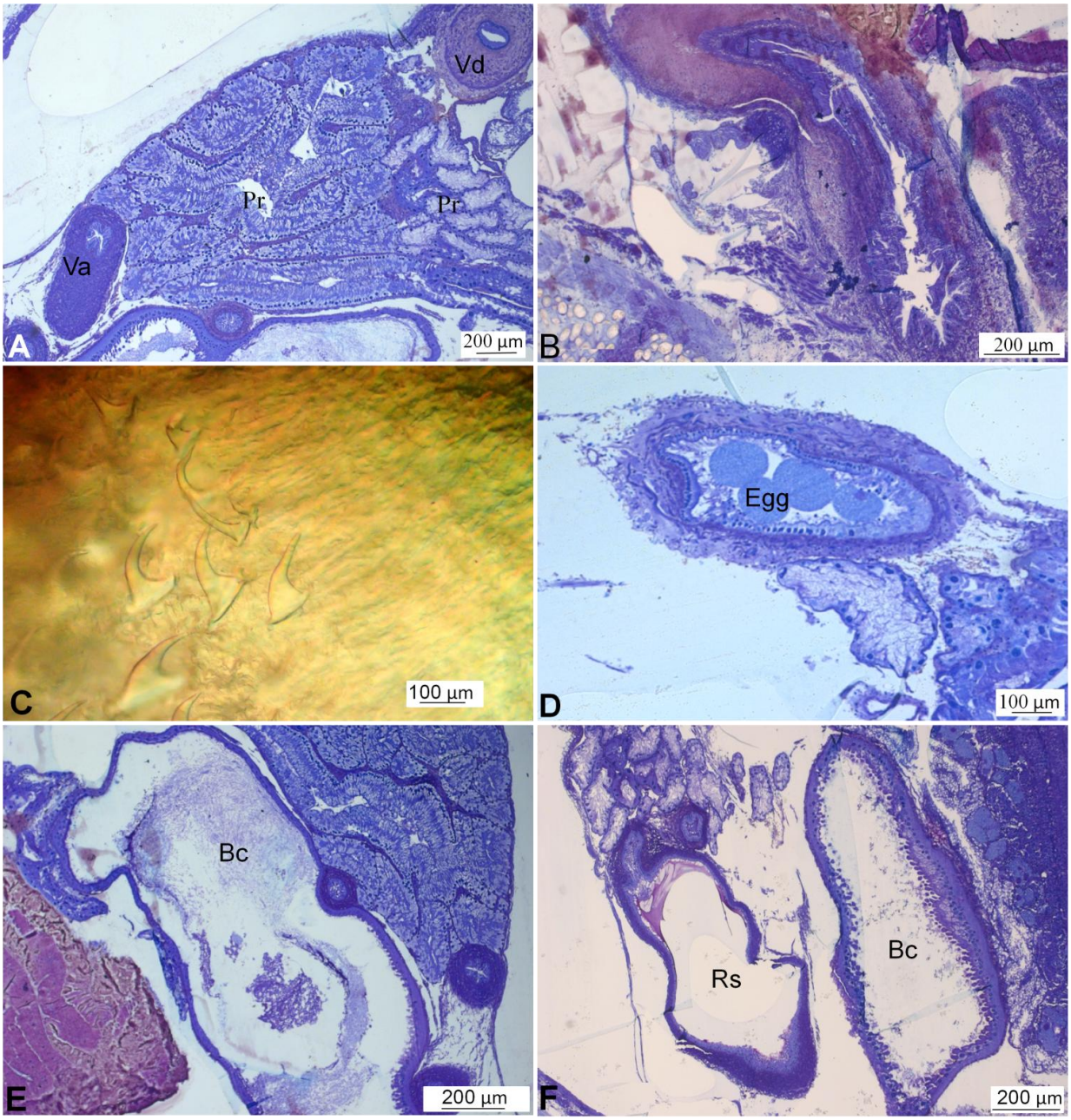

Fig. 9. Genital system of *Doriopsilla aroni* **sp. nov.**. A. Highly lobulated prostate gland. B. Transition of penis into penial sheath. C. Detail of penis showing the area, where spines start; note the various shapes. D. Fertilisation chamber containing a few eggs. E. Bursa copulatrix. F. Empty receptaculum seminis in vicinity of bursa copulatrix. Abbreviations: Bc = Bursa copulatrix; Pr = Prostate gland; Rs = Receptaculum seminis; Va = Vaginal duct; Vd = Vas deferens.

Additional features

Body cavity surrounded by a thick muscle layer, especially in the hind part of the body. One thick gill retractor starting close to gills and running ventrally towards anterior part of body. Small gill glands with pale bluish cells present at the base of gills, opening into gill pocket. A few unidentified parasites lying in the notal tissue, as well as close to the oral tube (Fig. 7B).

Phylogenetic analyses and species delimitation

Figs 10-12

Dendrodoris

Figs 10-12, Figs S1, S2

Phylogenetic analyses of the mitochondrial gene datasets (concatenated 16S and CO1, Fig. 10, 16S, Fig. 11, CO1 Fig. 12) including all three recognized genera of the family Dendrodorididae and several other dorid genera result in the paraphyly of the genus *Dendrodoris*, as members of the Phyllidiidae and other dorids cluster together with several *Dendrodoris* species. This is in contrast to the analyses of the nuclear gene datasets 18S and H3 where *Dendrodoris* is monophyletic (Fig. S1, S2).

Eleven *Dendrodoris* species cluster in the mitochondrial analyses with a bootstrap value of 100. This subclade is characterised by a long branch that separates it from all remaining members of the Dendrodorididae (Figs 10-12). The genetic distance of this long branch to other included dendrodorids ranges from approximately 45% (CO1: *D. limbata* to *D. citrina*) to 70% (group B to *D. krusensternii* (clade g) (Table S3)). These other dendrodorids always show a closer relationship to *Doriopsilla*, members of the Phyllidiidae or other dorid species, than with members of the long branch (Figs 10-12).

The ABGD test identifies 11 groups within *Dendrodoris* 16S sequences. The groups represent recognised species, but *D. temarana, D. grandiflora, D. senegalensis*, and *D. herytra* are united into one clade (Fig. 11) (group A, Table S2). *D. fumata* (only our sequences available) and *D. nigra* are distinct from all other *Dendrodoris* species (Fig. 11). Intraspecific variability in 16S sequences is less than 6% and interspecific variability ranges from at least 5% (between *D.*

krebsii and group A) to more than 50% (between long- and short-branch clades) (Table S2). Both *D. nigra* from the Persian Gulf, Philippines, and Australia and *D. fumata* (only sequences of specimens from Persian Gulf available) have their shortest genetic distance to group A (20–24% and 10-13 %, respectively) (Table S2).

The ABGD test based on the *Dendrodoris* CO1 sequences identifies 22 groups with more pronounced distances than in the 16S analysis (compare Table S2 and S3). The single and apparent barcode gap for intraspecific variability in the CO1 dataset is less than 3% and interspecific variability is more than 11%. Acknowledged species are usually recognised as such, e.g., the recently resurrected *D. temarana* (see Galià-Camps *et al.* 2022), or even split into clades. This is the case for *D. nigra* (clades a-c), with specimens from the Persian Gulf (including our specimens) forming a separate species (clade c), the specimens from Japan, Hawaii, and Australia united in clade a, and specimens from Singapore (unidentified species, accession number: MN690568) and Philippine Islands united in clade b. These clades show an interspecific genetic distance of 11-13 %. *D. fumata* also splits into several clades: all specimens from the Persian Gulf (including our specimens), Gulf of Oman and those from the Red Sea are united into one clade (clade e), whereas the single specimen from Australia is considered a separate species (clade f). The genetic distance with 4-6 % (Table S3) is rather small between these two clades. Another clade (clade d) unites sequences assigned to *D. fumata* from Hawaii and probably Singapore, and sequences from Japanese specimens, the latter assigned to *D. rubra*. Here the genetic difference to clade e and f lies between 8-11 % and 9-10 % respectively. *D. krusensternii* is divided into five clades (g-k) (Fig. 12) with a genetic difference of 6-13 % between these clades.

Doriopsilla

Figs 10-12

The genus *Doriopsilla* is monophyletic in the analyses using separate mitochondrial and nuclear gene data sets, but is paraphyletic in the concatenated dataset (16S and CO1, Fig. 10) with the long branch of *Dendrodoris* nested within the genus. *Cariopsilla pharpa* (only one CO1 sequence available) groups with *Doriopsilla* species (Fig. 10, Fig. 12).

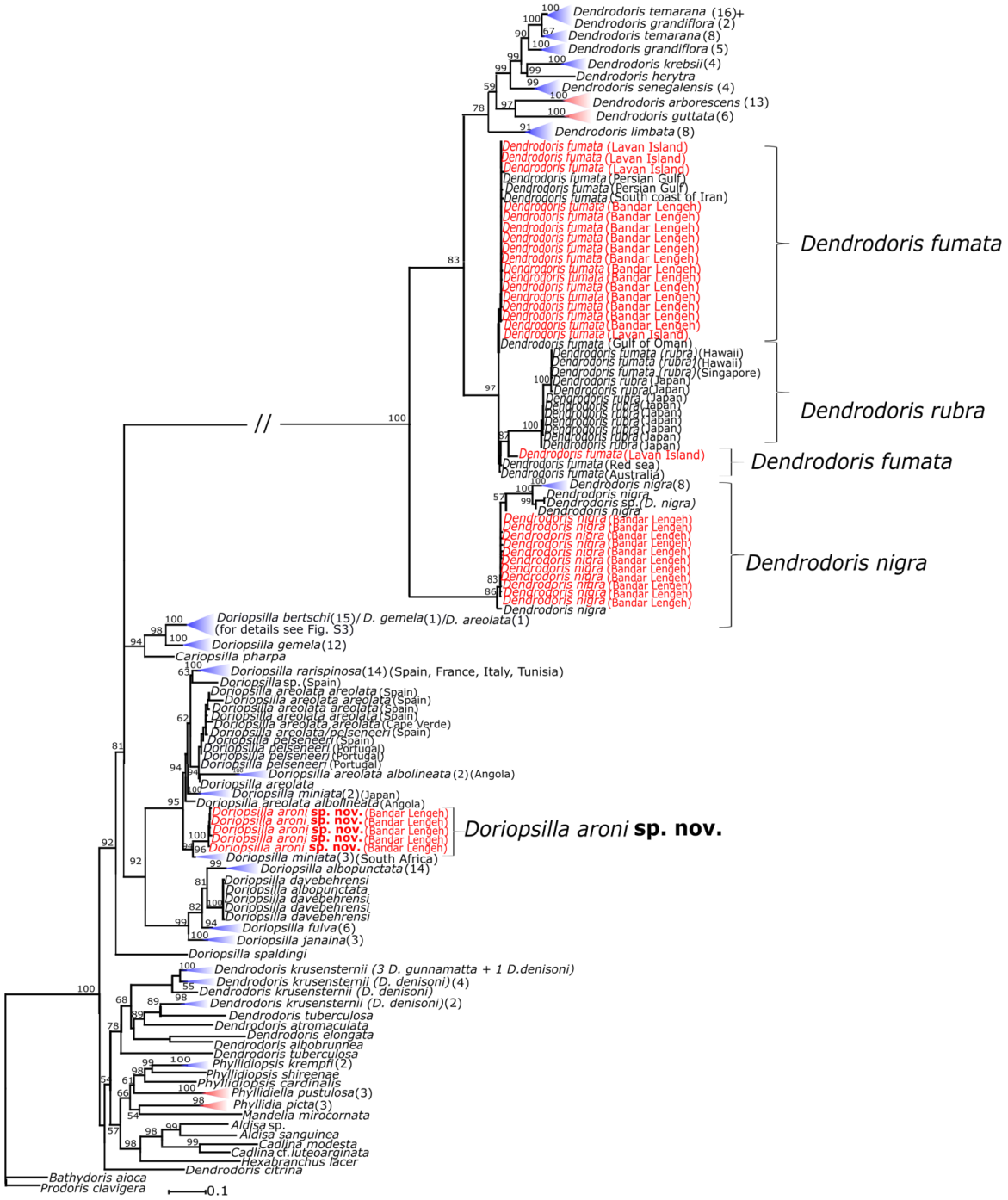

Fig. 10. Phylogenetic reconstruction of Dendrodorididae O'Donoghue, 1924 based on concatenated data set (CO1 and 16S), with *Bathydoris aioca* Er. Marcus & Ev. Marcus, 1962 and *Prodoris clavigera* (Thiele, 1912) as outgroup. Terminal taxa at species level partly collapsed (triangles coloured for clarity) and specimen numbers written in brackets. Species written in red are newly sequenced in this study. Numbers indicate bootstrap values for maximum likelihood (ML) test. Localities are provided for species closely related to the Iranian specimens.

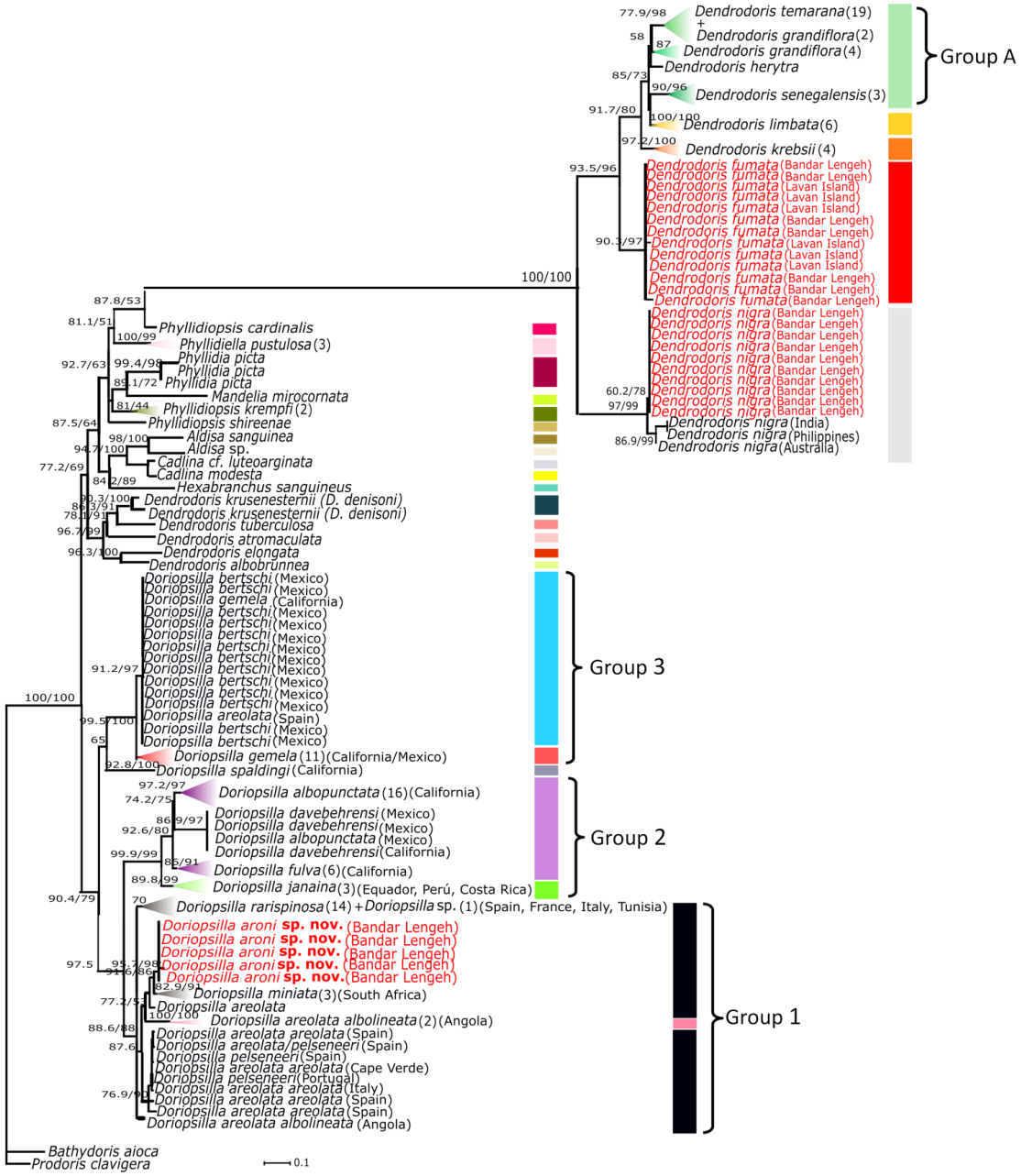

Fig. 11. Phylogenetic reconstruction of Dendrodorididae O'Donoghue, 1924 based on16S data set, with *Bathydoris aioca* Er. Marcus & Ev. Marcus, 1962 and *Prodoris clavigera* (Thiele, 1912) as outgroup. Terminal taxa at species level partly collapsed (triangles coloured for clarity) and specimen numbers written in brackets. Species in red are newly sequenced in this study. Species more related to the Iranian sequences are mentioned with their localities. Numbers before and after slash indicate approximate likelihood ratio test (SH-aLRT) and bootstrap values for maximum likelihood (ML) respectively. Values less than 50 are removed. Coloured bars indicate species delimitation resulting from ABGD test.

Doriopsilla aroni **sp. nov.** is not considered a separate species in the ABGD tests based on the 16S datasets (Fig. 11, Fig. S3), but form a clade (group 1) together with sequences from various species distributed or collected in the Mediterranean and the African West coast: *D. areolata, D. miniata, D. pelseneeri* d'Oliveira, 1895, *D. rarispinosa* Pruvot-Fol, 1951, and an unidentified *Doriopsilla* specimen from Spain (Fig. 11, black bar). The maximum genetic distance between the sequences within this group (group 1, Table 3) is 9 %. In this analysis also other *Doriopsilla* species are not recognized as distinct species and rather form larger clades with a genetic divergence between minimum of 17 % to maximum of 19% divergence (Fig S3, Table 3, groups 2 and 3). Only the single *Doriopsilla spaldingi* sequence is not united with other species.

Table 3. Intra- and interspecific pairwise uncorrected p-distances of *Doriopsilla* based on 16S data subset. Ranges between minimum and maximum distances are given as percentages. Each group comprises various species; Group 1: *D.* sp., *D. rarispinosa, D. areolata* and all its subspecies, *D. pelseneeri, D. miniata, Doriopsilla aroni* **sp. nov.**; Group 2: *D. albopunctata, D. fulva, D. davebehrensi, D. janaina;* Group 3: *D. gemela, D. bertschi* and one specimen of *D. areolata* from Spain which is probably misidentified.

	Group 1	Group 2	Group 3	*D. spaldingi*
Group 1	0-9			
Group 2	12-16	0-8		
Group 3	11-15	17-19	0-4	
D. spaldingi	12-16	16-18	13-14	0

ABGD tests using the CO1 data sets recognize our specimens from Iran as a separate species. Using the reduced *Doriopsilla* subset, 12 groups are identified with intraspecific variability less than 4% and interspecific variability between 5% -25 % (Table 4, Fig. S4). All species were recognised, including all the re-instated/re-investigated Mediterranean/Atlantic species by Furfaro *et al*. (2022). The gap between *Doriopsilla aroni* **sp. nov.** and the closely related species *D. rarispinosa*, *D. areolata*, *D. pelseneeri*, and *D. miniata* is 10-12%.

Table 4. Intra- and interspecific pairwise uncorrected p-distances of *Doriopsilla* based on the CO1 data subset including only *Doriopsilla*. Ranges between minimum and maximum distances are given as percentages.

	D. sp. (Spain)	*D. rarispinosa*	*D. areolata*	*D. pelseneeri*	*D. miniata*	*Doriopsilla aroni* **sp. nov.**	*D. gemela*	*D. bertschi*	*D. albopunctata*	*D. davebehrensi*	*D. fulva*	*D. spaldingi*
D. sp. (Spain)	0											
D. rarispinosa	10	0-2										
D. areolata	9-10	5-7	0									
D. pelseneeri	10	5-7	2-4	0-4								
D. miniata	11	6-7	7-8	6-8	0							
Doriopsilla aroni **sp. nov.**	13-14	10-11	11-12	10-12	11	0-1						
D. gemela	18-20	18-20	18-19	18-21	17-18	21-22	0-2					
D. bertschi	20	16-18	18	18-19	18	22	11-12	0				
D. albopunctata	24-25	21-23	20-22	19-22	20-22	20-23	15-18	17-18	0-3			
D. davebehrensi	24	19-21	21-23	21-23	21	22-23	16-17	17-18	8-10	0-1		
D. fulva	24-25	19-21	19-21	18-20	19-21	21-22	17-18	17-18	8-10	8-10	0-1	
D. spaldingi	22	20	22	21-22	19	23-24	16-18	17	21-22	18-19	20-21	0

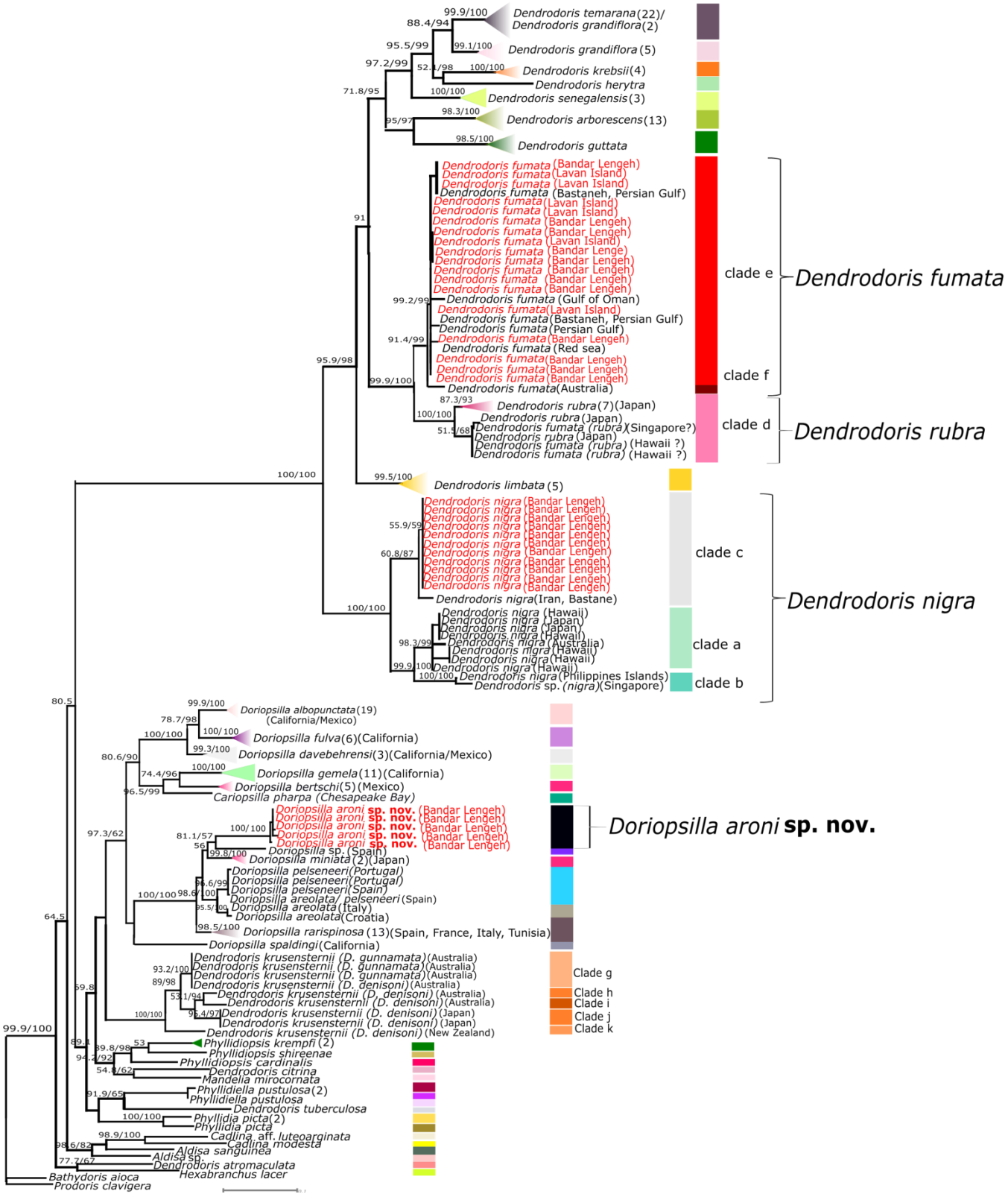

Fig. 12. Phylogenetic reconstruction of Dendrodorididae O'Donoghue, 1924 based on complete CO1 data set, with *Bathydoris aioca* Er. Marcus & Ev. Marcus, 1962 and *Prodoris clavigera*

(Thiele, 1912) as outgroup. Terminal taxa on species level partly collapsed (triangles coloured for better clarity) and specimen numbers written in brackets. Species written in red are new in this study. Species more related to the Iranian sequences are mentioned with their locality. Numbers before and after slash indicate approximate likelihood ratio test (SH-aLRT) and bootstrap values for maximum likelihood (ML) respectively. Values less than 50 are removed. Coloured bars indicate species delimitation resulting from ABGD test.

Doriopsilla aroni **sp. nov.** specimens from the Persian Gulf always form a monophyletic group (bootstrap support in concatenated, 16S, and CO1 analyses = 100, 98, 100, respectively). In the concatenated and 16S analyses (Figs 10, 11), the new species is the sister group of *Doriopsilla miniata* (bootstrap values 94 and 86, respectively). In the CO1 analysis, our specimens form a sister taxon to an undescribed *Doriopsilla* sp. from Spain, but with minor support (57), and these two are sister to *D. miniata* (Fig. 12).

Haplotype networking

The haplotype network analysis based on the CO1 subset (*Doriopsilla* species only) (Fig. 13) shows considerable distances between *Doriopsilla aroni* **sp. nov.** and all other *Doriopsilla* clades, also reflecting geographic distance (Fig. S5). None of the Iranian haplotypes are found in any other group, indicating that there is no link to other *Doriopsilla* populations. The closest relationship is to *D. rarispinosa*, *D. pelseneeri*, and *D. areolata* from the Mediterranean Sea, and also to two *D. miniata* from Japan. However, all these species are separated by many mutational steps from the Iranian specimens (Fig. 13). According to the rather linear network, gene flow between the different localities in the Mediterranean and Atlantic appears to be limited, resulting in these separate species. All species with a distribution along the Eastern Pacific coastline (*D. gemela*, *D. spaldingi*, *D. fulva*, *D. davebehrensi*, *D. albopunctata*, and *D. bertschi*; Fig. 13) are separated by many mutations from the Mediterranean/Indo Pacific species.

The haplotype network analysis based on the 16S dataset confirms partly the results of the CO1 analysis, but shows a more network structure than a linear one. Only few mutational steps separate the new species from species from the Mediterranean Sea, as well as *D. miniata* specimens from South Africa (Fig. 14, S6). *D. janaina* from the eastern South Pacific (Ecuador, Costa Rica, and Peru) shows its close relationship to the species *D. fulva*, *D. albopunctata*, and

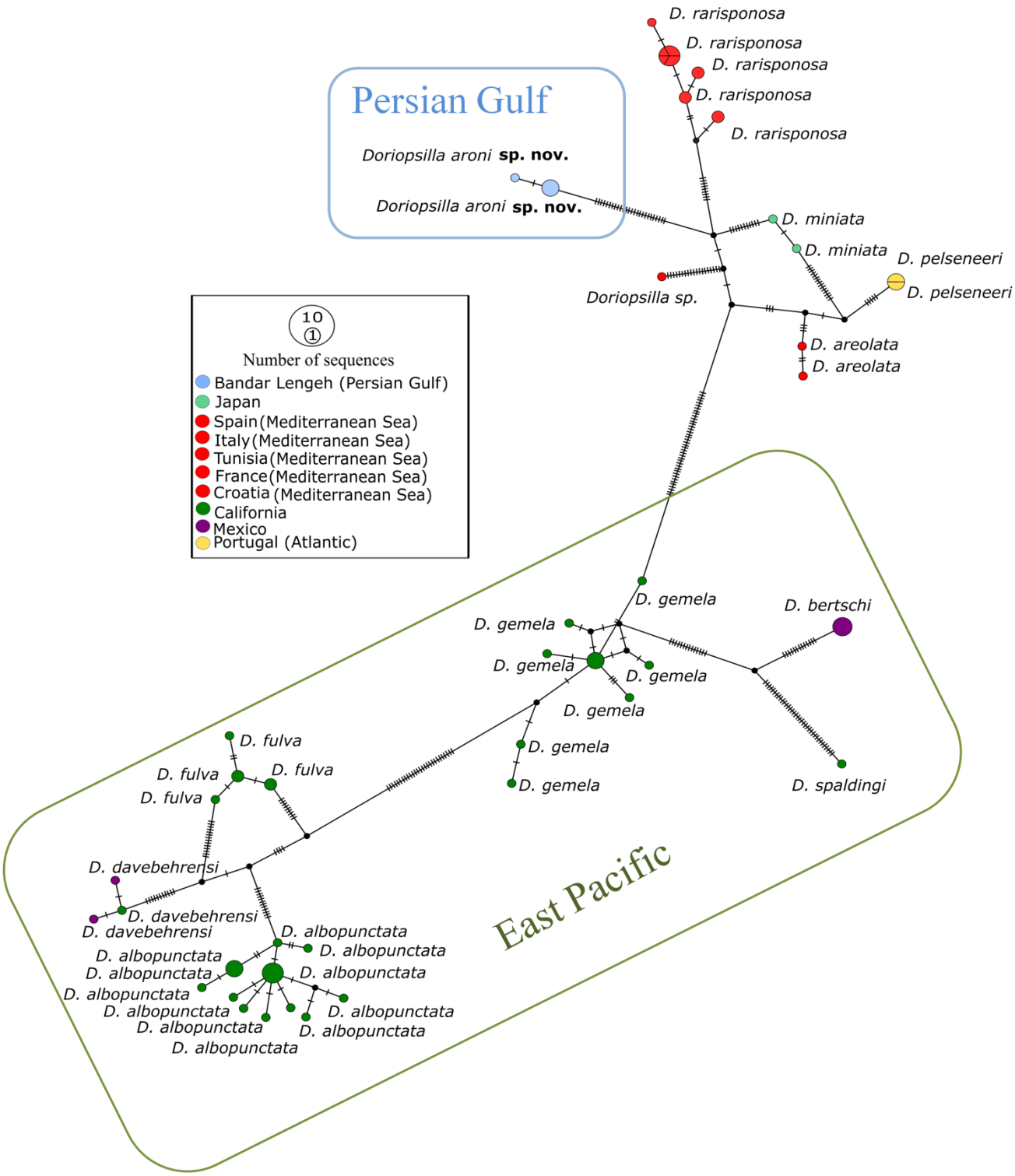

Fig. 13. Haplotype network of *Doriopsilla* Bergh, 1880 sequences from CO1 data subset (for the corresponding tree see Fig. S4). Coloured nodes on the haplotype network correspond to specific geographic localities and the size of the coloured nodes corresponds to the number of available sequences. Hash marks on the haplotype network denote mutational steps.

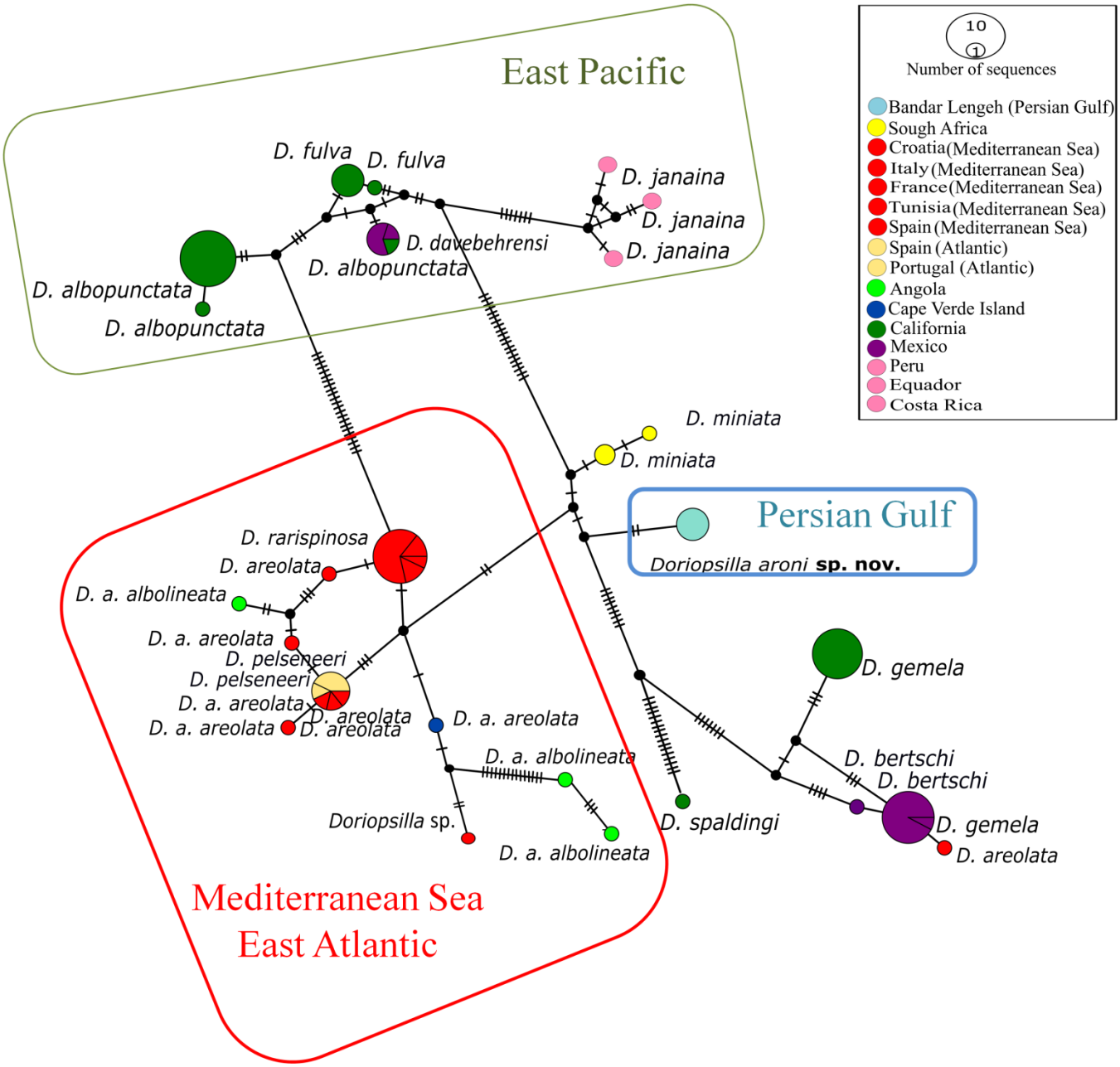

Fig. 14. Haplotype network of *Doriopsilla* Bergh, 1880 sequences from 16S data subset (for the corresponding tree see Fig. S3). Coloured nodes on the haplotype network correspond to specific geographic localities and the size of the coloured nodes corresponds to the number of analysed sequences. Hash marks on the haplotype network denote mutational steps.

D. davebehrensi from Mexico and California. However, in contrast to the CO1 analysis, *D. gemela* and *D. bertschi* from Mexico and California are separated from the sympatric species by many mutational steps. One specimen from Spain identified as *D. areolata* (see Valdés 2002) is

interesting, because it groups with the Mexican *D. bertschi* (Fig. 14). This could be a case of mistaken identity, but this does not explain why a Mediterranean specimen clusters with Pacific Ocean specimens.

Discussion

Molecular data

Hallas *et al*. (2017) and Korshunova *et al*. (2020) already indicated the paraphyly of the family Dendrodorididae, with *Doriopsilla* being more closely related to the Phyllidiidae than to the genus *Dendrodoris*. Our analyses are congruent with these results with an unresolved position of *Doriopsilla* according to the various gene datasets. Future studies including more nuclear genes, or even whole genomes, might be necessary to solve this conundrum.

Dendrodoris

Hallas *et al*. (2017) already recognised the divergence of certain *Dendrodoris* species (*D. nigra*, *D. fumata*, *D. arborescens*, and *D. guttata*) from other *Dendrodoris* species when applying mitochondrial genes. We identified here six further species which cluster in this long branch taxon. The reason for this unusually large genetic distance (more than 70 %) between a group of *Dendrodoris* species and the remaining Dendrodorididae, Phyllidiidae, and even other dorid species in our and other analyses could be explained by introgression, but certainly requires further investigation. Like the analysis of nuclear genes in dorids by Hallas *et al*. (2017), our investigation of the 18S and H3 dataset covering more of the taxa under consideration also recovered a monophyletic *Dendrodoris* clade.

Dendrodoris nigra has sometimes been considered a junior synonym of *D. fumata* (Gohar & Soliman 1967; Hirose *et al*. 2014). However, Brodie *et al*. (1997) make a clear distinction between these two species, and many subsequent studies follow her conclusions (e.g., Valdés & Gosliner 1999; Yonow 2012; Tibiriçá *et al*. 2017; Gosliner et al. 2018). Our molecular analysis on both mitochondrial genes confirms that *D. nigra* and *D. fumata* are two separate species. Nuclear genes are too conservative to solve the genus on species level. The mitochondrial gene 16S also seems quite conservative compared to the CO1 (minimum interspecific variability 5% and 11 % respectively) and does not recognize several of the acknowledged species. We concentrate therefore on the CO1 analysis, as was also done in former studies on dendrodorid

species (Galià-Camps *et al*. 2022, Furfaro *et al.* 2022) or other nudibranchs (e.g. Maroni & Wilson 2022 on the *Doris kerguelenensis* (Bergh, 1884) species complex). In view of the results from Galià-Camps *et al*. (2022), Ballesteros *et al.* (2023), and our CO1 analyses presented here, a re-examination of the widespread species *D. nigra* is necessary to address a putative cryptic variation. We can separate at least three different clades (a-c in Table S3) with a genetic difference of ~ 10 %, which is very close to the genetic difference between recognised species (e.g., 12 % between *D. krebsii* and *D. grandiflora*). Since the type locality of *D. nigra* lies in Japan, clade a combining specimens from Hawaii, Japan, and Australia, would therefore match best the distribution of the originally described *D. nigra*. However, before describing clade b from the Philippines and Singapore, and clade c with specimens from South coast of Iran as two new species, more sampling in the Indo-Pacific is needed for a better evaluation of the species status.

Until now, there has been some taxonomic confusion about *D. fumata* and *D. rubra*. Based on morphological studies Gohar & Soliman (1967) and Brodie *et al*. (1997) considered *D. rubra* a junior synonym of *D. fumata*. However, molecular analyses indicate that *D. rubra* is a valid species (Hirose *et al*. 2014; Fatemi *et al*. 2021). Our CO1 analyses confirm *D. fumata* and *D. rubra* as two distinct species in a sister group relationship. Two unpublished *D. fumata* sequences from Hawaii (accession numbers MW278338 and MW278039, registered by Paulay *et al*. (2020)), can be assigned to the species *D. rubra*. The type locality of *D. rubra* is in Trincomalee, along the North East coast of Sri Lanka (Kelaart 1858). Taking into account the localities of the so far available *D. rubra* sequences, this species is more distributed to the Western Pacific, whereas *D. fumata*, with its type locality in the Red Sea (Rüppell & Leuckart 1830) is more distributed in the Indic Ocean. Only one other sequence is available from Australia, which is considered as a separate species in our ABGD test (CO1 dataset, genetic divergence of 4-6 % to the other *D. fumata* sequences) and needs further investigation.

Most recently Galià-Camps *et al*. (2022) and Ballesteros *et al*. (2023) re-analysed the species complex *Dendrodoris grandiflora* and resurrected *D. temarana.* Their conclusions based on results obtained from various species delimitation tests using a subset of CO1 sequences from Mediterranean and Atlantic *Dendrodoris* species. Based on a genetic difference of 11% in our CO1 analysis, we can confirm these two as separate species. Two specimens assigned to *D. grandiflora* by Almanda *et al*. (2016) seem to be a misidentification and belong to *D. temarana*.

Dendrodoris krusensternii was originally described from Japan (see Valdés & Fahey 2006). Interestingly, our CO1 analysis identifies five clades, with partly disjunct distribution. The included two sequences from Japan are considered a separate species (clade j) from the three Australian clades (Clade g-i) and the single specimen from New Zealand (clade k). This indicates cryptic variation and therefore a re-investigation of *Dendrodoris denisoni* and *D. gunnamatta*, synonymized with *D. krusensternii* (Valdés & Fahey 2006, Nimbs & Smith 2021) is needed.

Doriopsilla

Despite the synonymization of the genus *Cariopsilla* with *Doriopsilla* by Valdés & Hamann (2008), *Doriopsilla pharpa* is still listed under the genus name *Cariopsilla* in the World Register of Marine Species (www.marinespecies.org/aphia.php?p=taxdetails&id=724107, last access 05/09/2023), Molluscabase (www.molluscabase.org/aphia.php?p=taxdetails&id=156711, last access 05/09/2023), or GBIF (www.gbif.org/species/7667786, last access 05/09/2023). The monotypic genus was created by Ortea & Espinosa (2006) in Espinosa *et al*. (2006) to take account of the special character of the tubercles covering the notum of *Doriopsilla pharpa*. We can confirm the conclusion of Valdés & Hamann (2008) that *Cariopsilla* should be considered a junior synonym of *Doriopsilla* and *Cariopsilla pharpa* is transferred back to the genus *Doriopsilla* as originally described by Er. Marcus (1961).

Few studies deal with the systematics of *Doriopsilla* species and species complexes. Most recently Furfaro *et al*. (2022) addressed the species complex *D. areolata* along the Mediterranean and south-east Atlantic coastline by analysing CO1 sequences, resulting in at least four different species with clear distribution boundaries: *D. areolata*, and *D. rarispinosa* within the Mediterranean Sea, *D. pelseneeri* and another probably undescribed species along the Mediterranean and Atlantic coast of Spain. Furfaro *et al*. (2022) also suggest that the more southern Atlantic specimens identified as *D. areolata* may represent *D. fedalae* Pruvot-Fol, 1953. Interestingly, several of the valid species differ in their reproductive mode, and in the structure of the egg masses (Hoover *et al*. 2015; Soares & Calado 2006; Valdés *et al*. 1996). Hoover *et al*. (2015) clarified the situation of the north-eastern Pacific species, resurrecting *D. fulva* and describing two new species, resulting in five species living sympatrically or in close geographical proximity. These studies contradict former analyses based on the more conservative mitochondrial gene 16S (Goodheart & Valdés 2013), which led to synonymisation of *Doriopsilla*

areolata species complex with the wide spread *D. miniata*. Our results of the species delimitation analyses using the 16S dataset also lead to lumping acknowledged species, thus indicating that 16S sequences again might be too conservative for species discrimination in Dendrodorididae. We therefore consider our CO1 analyses as more relevant for the discussion than the results on the 16S datasets. In the CO1 analyses our specimens from Persian Gulf are considered a distinct species, which in turn is more closely related to species from the Mediterranean or the eastern Atlantic. *Doriopsilla miniata* also shows a closer relationship, despite the long distance between their locations (west coast of South Africa (16S) and Japan (CO1) compared to the Persian Gulf). Unfortunately, no sequences from the type locality of *Doriopsilla miniata*, which lies at the south east coast of India, are available. But this species seems to be widespread in the Indo-Pacific (Goodheart & Valdés 2013; Gosliner *et al*. 2008; GBIF). It is very similar in colour to our specimens but is distinguished by a network of white lines on the notum, which is absent in our new species.

The sequence divergence between the new species and the closest related species (CO1: 10-14 %) is within the range or even greater than that between valid species. The lowest value is 5-7 % between *D. areolata, D. pelseneeri,* and *D. rarispinosa* from the Mediterranean/Atlantic region, and 6-7 % between *D. miniata* specimens from Japan and *D. rarispinosa* (Mediterranean region). Unfortunately, we have no sequences of the only other *Doriopsilla* species, which has so far been recorded exclusively from the Iranian coast, namely *D. nigrocera* Yonow, 2012. However, this species is characterised by diagnostic black rhinophores, a feature that is not present in our specimens, or in any other described species (Yonow 2012). Only one undescribed *Doriopsilla* species seems to have dark rhinophores, but this species is recorded from Papua New Guinea only (Gosliner *et al*. 2008; Gosliner *et al*. 2018). Probably, the three specimens mentioned from the south coast of the Persian Gulf (Kuwait) by Nithyanandan (2012) under the name *Doriopsilla* cf. *miniata*, also belong to our new species, as these specimens also lack the reticulated white lines on the mantle so characteristic of *D. miniata*.

Morphology

Dendrodoris

The morphology of *D. nigra*, which is widely distributed in the Indo Pacific and Red Sea (Tibiriçá *et al.* 2017; Gosliner *et al.* 2018; Yonow 2008) is well described (Brodie 2001; Valdés

et al. 1996; Valdés & Gosliner 1999; Wägele *et al*. 1999); however, these descriptions are mainly based on material from Australia or Hawaii. The only histological descriptions are based on material from Australia (Wägele *et al*. 1999), which was actually coming from the same collection as the *D. nigra* sequences from Australia included in our study here (Fig. 11, clade a in Fig. 12). Since the molecular analyses revealed cryptic variations in the species and consider the specimens from the Persian Gulf as a separate clade (clade c in Fig. 12), the analysis of the morphology/histology in comparison is of interest. The oral tube of the Iranian *D. nigra* specimen was surrounded by reddish stained glandular cells, a character not mentioned before in *D. nigra* descriptions, not even in the thorough histological study of Wägele *et al*. (1999). In our specimen, a sac-like stomach was observed, while Valdés (2002) mentioned an undifferentiated stomach for radula-less dorids, including *D. nigra*. Our specimen has spines on the penis, whereas the three specimens investigated by Wägele *et al.* (1999) lack these spines. Presence or absence of a cuticle is not mentioned in the latter study, but our specimen does not have a cuticle on the penis, in which the spines are embedded. Brodie *et al.* (1997) have already discussed the variation of penial spines in *Dendrodoris* species and therefore do not consider it a useful trait, as it can be influenced by ontogeny, but also by conservation. It cannot be excluded that the spines in the animals investigated by Wägele *et al*. (1999) (largest animal with 53 mm in length) were dissolved. The well developed and large nidamental glands, as well as the large vestibular gland indicate that our specimen was already fully mature, despite the small size of 19 mm. The smallest animal examined by Wägele *et al*. (1999) measured 23 mm and did not yet show any differentiation of the nidamental gland into capsule, membrane and mucous gland. Furthermore the vestibular gland was not yet developed either. These morphological characters support the molecular data that the clade c, comprising only specimens from the Persian Gulf, is a separate lineage from clade a, with specimens mainly of the Pacific Ocean.

Dendrodoris fumata is another well-studied species (Gohar & Soliman 1967; Brodie 2001; Brodie *et al.* 1997; Valdés & Gosliner 1999) with a wide distribution in the Indo-Pacific (e.g., Tibiriçá *et al.* 2017; Gosliner *et al*. 2018) and Red Sea (Yonow 2008). The species is also referred to in studies from Iran (Fatemi *et al*. 2021; Fatemi & Attaran 2015; Rezai *et al*. 2016). Fatemi *et al*. (2021) included a short external description (in Persian language) with an illustration of one *D. fumata* specimen, which matches our material. The presence or absence of penial hooks was considered to vary in the different colour forms of *D. fumata*: whereas the black

colour form seems to lack spines (Brodie *et al*. 1997), these hooks were described as present in the grey and red colour forms (Brodie *et al.* 1997; Ev. & Er. Marcus 1970; Edmunds 1971; Gohar & Soliman 1967; Eliot 1904) and this coincides with our reddish specimen that we investigated by histological means. Only Brodie (2001) provides some histological results on this species, mainly from the digestive tract. But she also described the vestibular gland for the first time for this species, a character that we confirm in our study. Interesting is the presence of a thick cuticle on the penis in our investigated specimen with a length of 23 mm. Future studies incorporating molecular data together with information on colour, size of specimens, and presence/absence of penial spines may reveal ontogenetic differences or the presence of cryptic variations not evident from previous studies.

Interesting is the description of symbiotic bacteria within the epithelium of the vestibular gland in *D. nigra*, a rather unique character (Klussmann-Kolb & Brodie 1999; Wägele *et al*. 1999). We also observed the typical fringe of microvilli housing the symbiotic bacteria in the vestibular gland in our investigated *D. nigra* and *D. fumata* specimens. We therefore can describe here for the first time the presence of bacteria in the vestibular gland of this species.

Doriopsilla

The genus *Doriopsilla*, comprising ~ 26 species, has a broad distribution in the temperate and tropical oceans of the Indian, Pacific, and Atlantic (see GBIF www.gbif.org/species/2292440, last access 06/09/2023). Only a few studies record *Doriopsilla* species from the Persian Gulf: A single specimen of an unidentified *Doriopsilla* species from the northern region of the Persian Gulf was mentioned by Rezaei *et al*. (2016). Yonow (2012) described a new species, *D. nigrocera*, from Jubail, Dahwat ad Daffi (eastern Saudi Arabia). The species description is based exclusively on the external description of one specimen and mentions the black rhinophores as a unique and distinguishing feature. The colour is similar to our specimens but the tubercles are covered in opaque white lines, which distinguishes *D. nigrocera* from our specimens in addition to the black rhinophores.

If we compare the geographical distribution of our specimens with the distribution of the 26 recognised species, and also take into consideration our results on the molecular data, we can exclude all species that only occur in the Atlantic and on the eastern Pacific coast: *D. albopunctata*, *D. bertschi*, *D. davebehrensi*, *D. elitae* Valdés & Hamann, 2008, *D. fulva*, *D.*

gemela, *D. rowena* Er. Marcus & Ev. Marcus, 1967, and *D. spaldingi*, from the eastern Pacific. *Doriopsilla espinosai* Valdés & Ortea, 1998, *D. evanae* Ballesteros & Ortea, 1980, *D. ciminoi* C. Ávila, Ballesteros & Ortea, 1992, *D. fedalae* Pruvot-Fol, 1953 (uncertain taxon), *D. nigrolineata*, and *D. tishae* Valdés & Hamann, 2008 are not considered further since they are described from the Caribbean Sea. *Doriopsilla pelseneeri* from Portugal has similarities with our *Doriopsilla* and will be discussed here, as are those species distributed throughout the Indo-Pacific.

Three *Doriopsilla* species have been described from South Australia: *D. aurea* (Quoy & Gaimard, 1832), *D. carneola* (Angas, 1864), and *D. peculiaris* (Abraham, 1877). *Doriopsilla aurea*, which reaches a length of 40 mm, is described as pinkish yellow-orange or red with some scattered white spots on the mantle (Burn 1962, 1966). Our specimens of *Doriopsilla aroni* **sp. nov.** lack these white spots.

Doriopsilla carneola was originally described as brown to orange, with small whitish mantle spots. Burn (1962, 1966) described other specimens with a maroon, dark red, or brown mantle. The rhinophores are red with a yellow to white base. The notum is covered with granules. Hence the species differs from our new species by the generally darker colour, and the presence of white spots on rather small tubercles (granules).

The colour of the *D. peculiaris* varies from white to orange. The stated length of up to 10 cm (Abraham 1877) exceeds the length of our specimens, which reached a maximum of 4 cm.

Doriopsilla debruini Perrone, 2001 from west coast of South Africa is externally characterised by a series of large dark brown spots on a pale brown mantle (Perrone 2001), in contrast to the yellow to red colour of *Doriopsilla aroni* **sp. nov.** With regards to the anatomy, *D. debruini* is described with probably few penis hooks ("hardly observed", Perrone 2001: 62), which is also incongruent with the well-developed spines on the penis of *Doriopsilla aroni* **sp. nov.**

Doriopsilla capensis Bergh, 1907, another species from the Atlantic coast of southern Africa, differs from our new species by its translucent white or cream to greenish colour with a narrow opaque white line around the mantle margin (Bergh 1907; Perrone 2001; Valdés & Gosliner 1999).

Doriopsilla pallida Bergh, 1902 was described from the Gulf of Thailand based on one preserved individual (6 mm in length; Bergh 1902). The colour of the living specimen is not known.

However, for the only specimen available to him, Bergh (1902) described the presence of ptyalin glands below the pharynx, a feature that is characteristic of the genus *Dendrodoris* and not of *Doriopsilla*.

According to our molecular data, *D. miniata,* whose original description is based on specimens east coast of India, is a closely related species to *Doriopsilla aroni* **sp. nov.** The length of our preserved specimens (up to 40 mm) is in a similar range as the *D. miniata* described by Alder & Hancock 1864 (35 mm) and the living specimens described by Gosliner *et al*. (2008) and Ávila *et al*. (1992) (30 and 40 mm). These authors as well as Marcus (1961) and Hirose *et al.* (2014) described *D. miniata* with different colours from pale orange to deep orange and always with a characteristic pattern of opaque white lines. However, the original description by Alder & Hancock (1864), which was based on preserved specimens, did not mention this white pattern. Since living specimens of *D. miniata* are always described with white lines (several specimens from India illustrated in GBIF have white lines and patterns), and taking into account the molecular results, we consider our specimens sufficiently distinguishable by the absence of the white lines on the notum except the tiny line around the gill pocket.

Doriopsilla aroni **sp. nov.** also differs from the Mediterranean species *D. areolata* and *D. rarispinosa*. Similar to *D. miniata*, *D. areolata* and *D. rarispinosa* show a typical white network on the notum, which was never observed in our five specimens of *Doriopsilla aroni* **sp. nov.** The only other species that lacks this white pigmentation in form of a network or lines and dots on the notum is *D. pelseneeri,* which, however, has a distribution restricted to the Atlantic shoreline of Spain and Portugal (Furfaro *et al.* 2022).

Further undescribed *Doriopsilla* species depictured in various biodiversity studies (e.g., Gosliner *et al*. 2008; Tibirica *et al*. 2017, Gosliner *et al*. 2018) usually have white lines or dots on the dorsal notum, and/or are recorded from localities rather far away from our type locality of D. aroni **sp. nov.**

Anatomical features are less suitable to delimit these different species (see also Valdés & Ortea 1997) as well as our new species. One feature that may differ is the armature of the penis. The penis of *D. areolata* is covered with numerous long and curved spines, which have a narrow base (Valdés & Ortea 1997). The size of the spines described in this paper is about 400 µm, and thus seem to be double the size as the spines in *D. aroni* **sp. nov.**, which however are similar in shape.

Bergh in his original description (1880) mentions the arrangement of the spines in ~ 15 longitudinal lines, which is similar to our new species. The spines of *D. pelseneeri* have a long base and straight cusp (Valdés & Ortea 1997), and clearly differ in their unusual shape. Pruvot-Fol (1951, 1954) depicted hooked spines in an irregular arrangement in *D. rarispinosa*, which differs from *D. aroni* **sp. nov.**.

Only one further study presents histological descriptions of a *Doriopsilla* species (Brodie 2001). She described the anterior part of the digestive tract and the parts of the genital system in detail for *Doriopsilla miniata* (specimen from New South Wales, Australia) and compared the results with three *Dendrodoris* species, including *D. fumata* (specimen from Queensland, Australia). *D. aroni* **sp. nov.** seems to have a less glandular oral tube, than described for *D. miniata*. A pyloric gland is mentioned for *D. pelseneeri*, but is missing in *D. areolata* (Valdés & Gosliner 1999). This structure has no glands, but a highly folded non glandular epithelium. The specimen that we investigated histologically was mature, indicating a recent copulation process. The receptaculum seminis was empty, indicating a transfer of the autosperm into a partner. Additionally, the prostate gland also showed areas of exuded contents. At the same time, eggs were still in the fertilization chamber.

Conclusions

Previous studies on Iranian marine heterobranch taxa show, that biodiversity studies in the Persian and Arabic Gulf reveal many species not recorded before from this area, but also new and probably endemic species (Amini-Yekta & Dekker 2021; Fatemi *et al*. 2021; Fatemi & Attaran 2015; Rezai *et al*. 2016; Maniei *et al*. 2020; Yonow 2012; Mousavipoor 2013; Nithyanandan 2012). Based on external features, but mainly on molecular results, we conclude that the *Doriopsilla* specimens collected along the Iranian shores cannot be assigned to any described *Doriopsilla* species, and thus represents a new species with distinct features. We can confirm the presence of at least two *Dendrodoris* species, *D. nigra* and *D. fumata*. However, the record of a third species, mentioned by Yonow (2012), with a possible assignment to *D. coronata*, still needs affirmation.

Our results confirm the most recent analysis of Furfaro *et al.* (2022) on validity of *D. rarispinosa*, and the endemic distribution of *D. areolata* s. str. in the Mediterranean Sea. However, with their study, as well as the studies of Galià-Camps et al. (2022) and ours, it seems that the CO1 marker

is more adapt for biodiversity studies in Dendrodoridídae. There are still many questions with regard to the cryptic diversification of *D. nigra*, or *D. krusensternii*. Histological investigations might provide evidencesfor specification and support results of molecular analyses, as we have shown here for a subclade of D. nigra. Finally, the reason, why mitochondrial genes split off a group of *Dendrodoris* species, resulting in a genetic divergence of more than 70 % is not understood and needs probably clarification by including mitochondrial and nuclear genomic analyses.

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Supplementary material:

Table S1. Data of Dendrodorididae sequences retrieved from NCBI GenBank with additional information on names when changed subsequently in literature (column 2), with locality information, references and accession numbers.

Species name taken from literature (NCBI) and names used in our ms	Subsequently in literature, WORMS or in this study corrected names	Locality	Reference	GenBank accession COI	GenBank accession 16S	GenBank accession 18S	GenBank accession H3
Cariopsilla pharpa	Assignment to *Doriopsilla* confirmed in this study	Chesapeake Bay (North Carolina)	Aguilar *et al*. 2018 (unpublished)	MH087486	-	-	-
Dendrodoris atromaculata		Philippines	Hallas *et al*. 2017	MF958434	MF958307	MF958348	-
Dendrodoris atromaculata		-	Donohoo & Gosliner (2020)	-	-	-	MN720315
Dendrodoris albobrunnea		New Caledonia	Valdés (2002)	-	AF430349	-	-
Dendrodoris arborescens		Japan	Hirose *et al*. 2014	AB917436	-	AB917459	-
Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917430	-	-	-
Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917431	-	-	-
Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917432	-	-	-
Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917433	-	-	-

Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917434	-	-	-
Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917435	-	-	-
Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917436	-	-	-
Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917437	-	-	-
Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917438	-	-	-
Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917439	-	-	-
Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917440	-	-	-
Dendrodoris arborenscens		Japan	Hirose *et al*. 2014	AB917441	-	-	-
Dendrodoris citrina		New Zealand	Shields (2009)	GQ292043	-	GQ326878	-
Dendrodoris denisoni	accepted as *D. krusensternii*	Japan	Hirose *et al*. 2014	AB917442	-	AB917460	-
Dendrodoris denisoni	accepted as *D. krusensternii*	Japan	Hirose *et al*. 2014	AB917443	-	-	-
Dendrodoris denisoni	accepted as *D. krusensternii*	New Zealand	Shields (2009)	GQ292047	-	GQ326872	-
Dendrodoris denisoni	accepted as *D. krusensternii*	New Caledonia	Valdés (2002)	-	AF430350	-	-
Dendrodoris denisoni	accepted as *D. krusensternii*	Philippines island	Hallas *et al*. 2017	-	MF958308	MF958349	-
Dendrodoris denisoni	accepted as *D. krusensternii*	Australia	Nimbs & Smith (2021)	MZ373328	-	-	-

D. denisoni in NCBI	accepted as *D. krusensternii*	Australia	Nimbs & Smith (2021)	MZ373329	-	-	-
D. denisoni in NCBI	*D. krusensternii* *(synonymized D. gunnamatta)*	Australia	Nimbs & Smith (2021)	MZ373327	-	-	-
D. denisoni in NCBI	*D. krusensternii* *(synonymized D. gunnamatta)*	Australia	Nimbs & Smith (2021)	MZ373326	-	-	-
D. denisoni in NCBI	*D. krusensternii* *(synonymized D. gunnamatta)*	Australia	Nimbs & Smith (2021)	MZ373325	-	-	-
D. denisoni in NCBI	accepted as *D. krusensternii*	Australia	Nimbs & Smith (2021)	MZ373324	-	-	-
Dendrodoris elongata		New Caledonia	Valdés (2002)	-	AF430351	-	-
Dendrodoris fumata		Iran (Gulf of Oman)	Mousavipoor (2013)	KF408220	-	-	-
Dendrodoris fumata		Iran (Persian Gulf)	Fatemi *et al*. 2021	MW402996	-	-	-
Dendrodoris fumata		Iran (Persian Gulf)	Fatemi *et al*. 2021	MW392739	-	-	-
Dendrodoris fumata		South coast line of Iran	Fatemi *et al*. 2020 (unpublished)	MN548840	-	-	-
Dendrodoris fumata	***D. rubra***	Hawaii (locality mentioned in title)	Paulay *et al*. 2017 (not published)	MW278338	-	-	-

Dendrodoris fumata	***D. rubra***	Hawaii (locality mentioned in title)	Paulay *et al*. 2017 (not published)	MW278039	-	-	-
Dendrodoris fumata	***D. rubra***	Singapore	Ip e*t al*. 2019	MN690567	-	-	-
Dendrodoris fumata		Australia	Wollscheid-Lengeling *et al*. 2001	AF249799	-	-	-
Dendrodoris fumata		Saudi Arabia (Red sea)	Hallas *et al*. 2017	MF958444	-	MF958358	-
Dendrodoris fumata		Australia	Wollscheid-Lengeling *et al*. 2001	-	-	AF249216	-
Dendrodoris fumata		-	Göbbeler & Klussmann-Kolb (2010)	-	-	FJ917444	-
Dendrodoris rubra		Japan	Hirose *et al*. 2014	AB917448	-	-	-
Dendrodoris rubra		Japan	Hirose *et al*. 2014	AB917449	-	-	-
Dendrodoris rubra (fumata)		Japan	Hirose *et al*. 2014	AB917450	-	AB917463	-
Dendrodoris rubra		Japan	Hirose *et al*. 2014	AB917451	-	-	-
Dendrodoris rubra		Japan	Hirose *et al*. 2014	AB917452	-	-	-
Dendrodoris rubra		Japan	Hirose *et al*. 2014	AB917453	-	-	-
Dendrodoris rubra		Japan	Hirose *et al*. 2014	AB917454	-	-	-

Dendrodoris rubra		Japan	Hirose *et al*. 2014	AB917455	-	-	-
Dendrodoris rubra		Japan	Hirose *et al*. 2014	AB917456	-	-	-
Dendrodoris grandiflora		Spain	Almanda *et al*. 2016	KT833268	KT820538	-	-
Dendrodoris grandiflora		Spain	Almanda *et al*. 2016	KT833269	KT820539	-	-
Dendrodoris grandiflora		Spain	Galia-Camps *et al*. 2022	MW194018	MW194921	-	MW200264
Dendrodoris grandiflora		Spain	Galia-Camps *et al*. 2022	MW194016	MW194922	-	MW200262
Dendrodoris grandiflora		Spain	Galia-Camps *et al*. 2022	MW194013	-	-	MW200257
Dendrodoris grandiflora		Spain	Galia-Camps *et al*. 2022	MW194012	MW194925	-	MW200256
Dendrodoris grandiflora		Spain	Galia-Camps *et al*. 2022	MW194011	MW194926	-	MW200255
Dendrodoris guttata		Japan	Hirose *et al*. 2014	AB917444	-	-	-
Dendrodoris guttata		Japan	Hirose *et al*. 2014	AB917446	-	AB917461	-
Dendrodoris guttata		Japan	Hirose *et al*. 2014	AB917445	-	-	-
Dendrodoris guttata		Japan	Hirose *et al*. 2014	AB917446	-	-	-
Dendrodoris guttata		Korea	Park J. 2018 (unpublished)	MG948856	-	-	-
Dendrodoris guttata		Korea	Park J. 2018 (unpublished)	MG948855	-	-	-
Dendrodoris herytra		Spain	Galia-Camps *et al*. 2022	MW194025	MW194915	-	MW200270

Dendrodoris krebsii		Cuba	Galia-Camps *et al.* 2022	MW194028	MW194912	-	MW200273
Dendrodoris krebsii		Cuba	Galia-Camps *et al.* 2022	MW194027	MW194913	-	MW200272
Dendrodoris krebsii		Cuba	Galia-Camps *et al.* 2022	-	MW194911	-	MW200274
Dendrodoris krebsii		Cuba	Galia-Camps *et al.* 2022	MW194026	MW194914	-	MW200271
Dendrodoris limbata		Spain	Galia-Camps *et al.* 2022	-	MW194898	-	-
Dendrodoris limbata		Spain	Galia-Camps *et al.* 2022	-	MW194897	-	-
Dendrodoris limbata		Spain	Galia-Camps *et al.* 2022	-	MW194901	-	-
Dendrodoris limbata		Italy	Galia-Camps *et al.* 2022	MW194032	-	-	MW200277
Dendrodoris limbata		Spain	Galia-Camps *et al.* 2022	MW194031	MW194908	-	MW200276
Dendrodoris limbata		Spain	Galia-Camps *et al.* 2022	MW194030	MW194909	-	-
Dendrodoris limbata		Spain	Galia-Camps *et al.* 2022	MW194029	MW194910	-	MW200275
Dendrodoris limbata		Italy	Galia-Camps *et al.* 2022	MW194009	-	-	MW200253
Dendrodoris nigra		Australia	Wollscheid-Lengeling *et al.* 2001	AF249795	AF249242	AF249215	-
Dendrodoris nigra		Philippines Island	Hallas *et al.* 2017	MF958443	MF958318	MF958357	-
Dendrodoris nigra		Japan	Hirose *et al.* 2014	AB917447	-	AB917462	-
Dendrodoris nigra		Japan	Ah Shee Tee 2019 (unpublished)	MN168888	-	-	-

Dendrodoris nigra		probably India	Dixit *et al.* 2021 (unpublished)	-	MT592807	-	-
Dendrodoris nigra		south Coast line, Iran	Fatemi *et al*. 2020 (unpublished)	MN548839	-	-	-
Dendrodoris nigra		Hawaii	Paulay *et al*. 2017 (not published)	MW277667		-	-
Dendrodoris nigra		Hawaii	Paulay *et al*. 2017 (not published)	MW277935	-	-	-
Dendrodoris nigra		Hawaii	Paulay *et al*. 2017 (not published)	MW277927	-	-	-
Dendrodoris nigra		Hawaii	Paulay *et al*. 2017 (not published)	MW278028	-	-	-
Dendrodoris nigra		Hawaii	Paulay *et al*. 2017 (not published)	MW277982	-	-	-
Dendrodoris nigra		-	Shields (2009)	-	-	GQ326871	
Dendrodoris sp.	*D. nigra*	Singapore	Ip e*t al*. 2019	MN690568	-	-	-
Dendrodoris tuberculosa		Korea	Cheney *et al*. 2014	KJ001303	-	-	-
Dendrodoris tuberculosa		New Caledonia	Valdés (2002)	-	AF430352	-	-
Dendrodoris senegalensis		Cabo Verde	Galia-Camps *et al.* 2022	MW194039	-	-	MW200278

Dendrodoris senegalensis		Cabo Verde	Galia-Camps *et al.* 2022	-	MW194905	-	MW200280
Dendrodoris senegalensis		Cabo Verde	Galia-Camps *et al.* 2022	MW194038	MW194907	-	MW200286
Dendrodoris senegalensis		Cabo Verde	Galia-Camps *et al.* 2022	MW194033	MW194906	-	MW200279
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	-	-	-	MW200260
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194043	MW194894	-	MW200267
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194042	MW194895	-	-
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194041	MW194896	-	MW200288
Dendrodoris temarana		Cabo Verde	Galia-Camps *et al.* 2022	MW194040	-	-	MW200287
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194037	MW194899	-	MW200265
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194036	MW194900	-	MW200285
Dendrodoris temarana		Cabo Verde	Galia-Camps *et al.* 2022	MW194035	MW194902	-	MW200283
Dendrodoris temarana		Cabo Verde	Galia-Camps *et al.* 2022	MW194034	MW194903	-	MW200282
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194024	MW194916	-	MW200269
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194023	MW194917	-	MW200290
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194022	MW194918	-	MW200268
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194021	MW194919	-	MW200289
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194020	MW194920	-	MW200258

Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194019	-	-	MW200284
Dendrodoris temarana		Portugal	Galia-Camps *et al.* 2022	MW194017	-	-	MW200263
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194015	-	-	MW200259
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194014	MW194924	-	MW200266
Dendrodoris temarana		Spain	Galia-Camps *et al.* 2022	MW194010	MW194927	-	MW200254
Dendrodoris temarana		Cabo Verde	Galia-Camps *et al.* 2022	-	MW194904	-	MW200281
Dendrodoris temarana		Portugal	Galia-Camps *et al.* 2022	-	MW194923	-	MW200261
Dendrodoris temarana		Morocco	Galia-Camps *et al.* 2022	MZ710315	MZ429957	-	MZ713159
Dendrodoris temarana		Morocco	Galia-Camps *et al.* 2022	MZ710316	MZ429958	-	MZ713160
Dendrodoris temarana		Morocco	Galia-Camps *et al.* 2022	MZ710317	MZ429959	-	MZ713161
Dendrodoris temarana		Morocco	Galia-Camps *et al.* 2022	MZ710318	MZ429960	-	MZ713162
Doriopsilla albopunctata		Mexico	Valdés (2002)	-	AF430354	-	-
Doriopsilla albopunctata		California	Wetzer *et al.* 2020 (not published)	MK550636	-	-	-
Doriopsilla albopunctata		California	Hoover *et al.* 2015	KR002483	-	-	KR002525
Doriopsilla albopunctata		California	Hoover *et al.* 2015	KR002480	KR002428	-	-
Doriopsilla albopunctata		California	Hoover *et al.* 2015	KR002481	KR002429	-	KR002524
Doriopsilla		California	Hoover *et al.*	KR002482	KR002430	-	-

albopunctata			2015				
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002485	KR002431	-	KR002527
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002486	KR002432	-	KR002528
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002487	KR002433	-	-
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002488	KR002434	-	KR002529
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002489	KR002435	-	KR002530
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002490	KR002436	-	KR002531
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002491	KR002437	-	KR002532
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002492	KR002438	-	KR002533
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002493	KR002439	-	KR002534
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002494	KR002440	-	KR002535
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002495	KR002441	-	KR002536
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002496	KR002442	-	KR002537
Doriopsilla albopunctata		California	Hoover *et al*. 2015	KR002497	KR002443	-	KR002538
Doriopsilla areolata		Croatia	Furfaro *et al.* 2022	ON211997	ON229526	-	ON209460
Doriopsilla areolata		Italy	Furfaro *et al.* 2022	ON211996	ON229532	-	ON209466
Doriopsilla areolata		Spain	Valdés (2002)	-	AF430355	-	-

Doriopsilla areolata		Spain?	Almanda *et al.* 2016	-	KT820537	-	-
Doriopsilla areolata	*(Doriopsilla pelseneeri* (Furfaro *et al*. 2022))	Andalusia, Spain (Mediterranean)	Thollesson (2000)	AJ223262	AJ225186	-	-
Doriopsilla areolata areolata		Cape Verde	Goodheart & Valdés (2013)	-	KC171027	-	-
Doriopsilla areolata areolata		Cádiz, Spain (Atlantic)	Goodheart & Valdés (2013)	-	KC171025	-	KC171036
Doriopsilla areolata areolata		Girona, Spain(Mediterranean)	Goodheart & Valdés (2012)	-	KC171023	-	KC171040
Doriopsilla areolata areolata		Cádiz, Spain (Atlantic)	Goodheart & Valdés (2013)	-	KC171024	-	KC171035
Doriopsilla areolata areolata		Las palmas, Spain	Goodheart & Valdés (2012)	-	KC171026	-	KC171037
Doriopsilla areolata albolineata		Angola	Goodheart & Valdés (2013)	-	KC171033	-	KC171039
Doriopsilla areolata albolineata		Angola	Goodheart & Valdés (2013)	-	KC171032	-	-
Doriopsilla areolata albolineata		Angola	Goodheart & Valdés (2013)	-	KC171031	-	KC171038
Doriopsilla bertschi		Mexico	Hoover *et al.* 2015	KR002517	KR002462	-	KR002553
Doriopsilla bertschi		Mexico	Hoover *et al.* 2015	KR002518	KR002463	-	KR002554
Doriopsilla bertschi		Mexico	Hoover *et al.* 2015	KR002519	KR002472	-	KR002549
Doriopsilla bertschi		Mexico	Hoover *et al.* 2015	-	KR002464	-	-

Doriopsilla bertschi		Mexico	Hoover *et al*. 2015	-	KR002465	-	-
Doriopsilla bertschi		Mexico	Hoover *et al*. 2015	-	KR002466	-	KR002555
Doriopsilla bertschi		Mexico	Hoover *et al*. 2015	-	KR002467	-	-
Doriopsilla bertschi		Mexico	Hoover *et al*. 2015	-	KR002468	-	-
Doriopsilla bertschi		Mexico	Hoover *et al*. 2015	-	KR002469	-	-
Doriopsilla bertschi		Mexico	Hoover *et al*. 2015	-	KR002470	-	KR002559
Doriopsilla bertschi		Mexico	Hoover *et al*. 2015	-	KR002471	-	-
Doriopsilla bertschi		Mexico	Hoover *et al*. 2015	-	KR002473	-	-
Doriopsilla bertschi		Mexico	Hoover *et al*. 2015	-	KR002474	-	KR002557
Doriopsilla bertschi		Mexico	Hoover *et al*. 2015	KR002515	-	-	-
Doriopsilla bertschi		Mexico	Hoover *et al*. 2015	KR002516	-	-	-
Doriopsilla davebehrensi		Mexico	Hoover *et al*. 2015	KR002520	KR002475	-	KR002564
Doriopsilla davebehrensi		Mexico	Hoover *et al*. 2015	KR002521	KR002476	-	KR002564
Doriopsilla davebehrensi		California	Hoover *et al*. 2015	KR002522	KR002477	-	-
Doriopsilla davebehrensi		Mexico	Hoover *et al*. 2015	-	KR002478	-	KR002566
Doriopsilla gemela *(D. areolata* based on hoover *et al. 2015)*		Mexico	Valdés (2002)	-	AF430356	-	-

Doriopsilla fulva		California	Hoover *et al*. 2015	KR002499	KR002445	-	KR002540
Doriopsilla fulva		California	Hoover *et al*. 2015	KR002500	KR002446	-	-
Doriopsilla fulva		California	Hoover *et al*. 2015	KR002501	KR002447	-	-
Doriopsilla fulva		California	Hoover *et al*. 2015	KR002502	KR002448	-	-
Doriopsilla fulva		California	Hoover *et al*. 2015	KR002503	KR002449	-	KR002541
Doriopsilla fulva		California	Hoover *et al*. 2015	KR002498	KR002444	-	KR002539
Doriopsilla gemela		California	Hoover *et al*. 2015	KR002504	KR002451	-	KR002543
Doriopsilla gemela		California	Hoover *et al*. 2015	KR002450	KR002542	-	KR002542
Doriopsilla gemela		California	Hoover *et al*. 2015	KR002505	KR002452	-	KR002542
Doriopsilla gemela		California	Hoover *et al*. 2015	KR002506	KR002453	-	KR002544
Doriopsilla gemela		California	Hoover *et al*. 2015	KR002507	KR002454	-	KR002545
Doriopsilla gemela		California	Hoover *et al*. 2015	KR002508	KR002455	-	KR002546
Doriopsilla gemela		California	Hoover *et al*. 2015	KR002509	KR002456	-	KR002547
Doriopsilla gemela		California	Hoover *et al*. 2015	KR002510	KR002457	-	KR002548
Doriopsilla gemela		California	Hoover *et al*. 2015	KR002511	KR002458	-	KR002549
Doriopsilla gemela		California	Hoover *et al*. 2015	KR002512	KR002459	-	KR002550
Doriopsilla gemela		California	Hoover *et al*. 2015	KR002513	KR002460	-	KR002551

Doriopsilla gemela		California	Hoover *et al*. 2015	KR002514	KR002461	-	KR002552
Doriopsilla janaina		Equador	Hallas *et al*. 2017	-	MF958312	MF958353	-
Doriopsilla janaina		Perú	Goodheart & Valdés (2012)	-	KC171022	-	KC171034
Doriopsilla janaina		Costa Rica	Valdés (2002)	-	AF430357	-	-
Doriopsilla miniata		Japan	Hirose *et al*. 2014	AB917457	-	AB917464	-
Doriopsilla miniata		Japan	Hirose *et al*. 2014	AB917458	-	-	-
Doriopsilla miniata		South Africa, Atlantic	Goodheart & Valdés (2013)	-	KC171030	-	KC171043
Doriopsilla miniata		South Africa, Atlantic	Goodheart & Valdés (2013)	-	KC171029	-	KC171042
Doriopsilla miniata		South Africa, Atlantic	Goodheart & Valdés (2013)	-	KC171028	-	KC171041
Doriopsilla pelseneeri		Berlengas, Portugal (Atlantic)	Almanda *et al*. 2016	KT833267	-	-	-
Doriopsilla pelseneeri		Berlengas, Portugal (Atlantic)	Almanda *et al*. 2016	KT833266	-	-	-
Doriopsilla pelseneeri		Andalusia, Spain (Mediterranean)	Furfaro *et a*l. 2022	ON211995	ON229525	-	ON209459
Doriopsilla pelseneeri		Berlengas, Portugal (Atlantic)	Almanda *et al*. 2016	-	KT820536	-	-

Doriopsilla rarispinosa		Italy	Furfaro *et al*. 2022	ON211998	ON229527	-	ON209461
Doriopsilla rarispinosa		Italy	Furfaro *et al*. 2022	ON211999	ON229528	-	ON209462
Doriopsilla rarispinosa		Italy	Furfaro *et al*. 2022	ON212000	ON229529	-	ON209463
Doriopsilla rarispinosa		Italy	Furfaro *et al*. 2022	ON212001	ON229530	-	ON209464
Doriopsilla rarispinosa		Italy	Furfaro *et al*. 2022	ON212003	ON229533	-	ON209467
Doriopsilla rarispinosa		Italy	Furfaro *et al*. 2022	ON212004	ON229534	-	ON209468
Doriopsilla rarispinosa		Italy	Furfaro *et al*. 2022	ON212005	ON229535	-	ON209469
Doriopsilla rarispinosa		Italy	Furfaro *et al*. 2022	ON212006	ON229536	-	ON209470
Doriopsilla rarispinosa		Tunisia	Furfaro *et al*. 2022	ON212002	ON229531	-	ON209465
Doriopsilla rarispinosa		France (mediterranean)	Furfaro *et al*. 2022	ON212007	ON229537	-	ON209471
Doriopsilla rarispinosa		France (mediterranean)	Furfaro *et al*. 2022	ON212008	ON229538	-	ON209472
Doriopsilla rarispinosa		Franc (mediterranean)	Furfaro *et al*. 2022	-	ON229539	-	ON209473
Doriopsilla rarispinosa		Catalonia, Spain (mediterranean)	Furfaro *et al*. 2022	ON212009	ON229540	-	ON209474
Doriopsilla rarispinosa		Catalonia, Spain (mediterranean)	Furfaro *et al*. 2022	ON212010	ON229541	-	ON209475

Doriopsilla sp. 1		Andalusia, Spain (Mediterranean)	Furfaro *et al.* 2022	ON211994	ON229524	-	ON209458
Doriopsilla spaldingi		California	Hoover *et al*. 2015	KR002479	KR002427	-	KR002523
Aldisa sanguinea		California	Hallas *et al*. 2017	MF958435	MF958309	MF958350	-
Aldisa sp.		Malaysia	Hallas *et al*. 2017	MF958436	EU982818	MF958351	-
Aldisa zavorensis		-	Hallas *et al*. 2017	-	-	-	MF327391
Aldisa fragaria		-	Hallas *et al*. 2017	-	-	-	MF327390
Aldisa fragaria		-	Hallas *et al*. 2017	-	-	-	MF327389
Aldisa smaragdina		-	Oskars *et al*. 2015	-	-	-	KJ022914
Aldisa albatrossae		-	Mahguib & Valdés (2015)	-	-	-	KP871655
Bathydoris aioca			Mahguib & Valdés (2015)	KP871635	KP871682		
Cadlina aff. *luteomarginata*		Scotland	Hallas *et al*. 2017	KM219678	KJ653679	-	KM225828
Cadlina modesta		California	Hallas *et al*. 2017	MF958437	MF958310	-	-
Hexabranchus lacer		-	Hallas *et al*. 2017, Tibiriçá *et al*. 2023	MF958433	MF958305	-	-
Mandelia mirocornata		-	Mahguib & Valdés (2015)	KP871646	KP871694	-	-
Phyllidia picta		-	Undap *et al.* 2019	MN248542	MN217672	-	-

Phyllidia picta		-	Undap *et al.* 2019	MN248545	MN217674	-	-
Phyllidia picta		-	Undap *et al.* 2019	MN248546	MN217675	-	-
Phyllidia coelestis		-	Wollscheid-Lengeling *et al*. 2001	-	-	AF249209	-
Phyllidia flava		-	Furfaro *et al*. 2022	-	-	-	ON209476
Phyllidia larryi		-	Mahguib & Valdés (2015)	-	-	-	KP871672
Phyllidiella pustulosa		-	Undap *et al.* 2019	MN248608	MN243996	-	-
Phyllidiella pustulosa		-	Undap *et al.* 2019	MN248609	MN243997	-	-
Phyllidiella pustulosa		-	Undap *et al.* 2019	MN248607	MN243995	-	-
Phyllidiella pustulosa		-	Wollscheid-Lengeling *et al*. 2001	-	-	AF249208	-
Phyllidiella nigra		-	Hallas *et al.* 2017	-	-	MF958322	-
Phyllidiopsis krempfi		-	Undap *et al.* 2019	MN248649	MN244072	-	-
Phyllidiopsis krempfi		-	Undap *et al.* 2019	MN248646	MN244070	-	-
Phyllidiopsis shireenae		-	Undap *et al.* 2019	MN248659	MN244082	-	-
Phyllidiopsis cardinalis		-	Cheney *et al*. 2014	KJ001308	-	-	-
Phyllidiopsis cardinalis		-	Valdés (2002)	-	AF430367	-	-
Phyllidiopsis annae		-	Hallas *et al.* 2017	-	-	MF958324	-

Prodoris clavigera		-	Hallas *et al.* 2017	-	-	AY165754	-
Prodoris clavigera		-	Hallas *et al.* 2017	-	-	MF958320	-
Prodoris clavigera		-	Ortigosa *et al.* 2017	-	-	-	MK474134

Table S2. Intra- and interspecific pairwise uncorrected p-distances of *Dendrodoris* based on 16S data set. Ranges between minimum and maximum distances are given as percentages. Group A: *D. temarana, D. grandiflora, D. senegalensis* and *D. herytra.*

	D. nigra	Group A	*D. krebsii*	*D. limbata*	*D. fumata*	*D. tuberculosa*	*D. krusensternii*	*D. krusensternii*	*D. elongata*	*D. albobrunnea*	*D. atromaculata*
D. nigra	0-4										
Group A	20-24	0-6									
D. krebsii	22-23	5-8	0								
D. limbata	23-25	6-8	0-10	0							
D. fumata	21-24	10-13	12	14	0-1						
D. tuberculosa	51-58	49-55	58	56	58-59	0					
D. krusensternii	48-51	49-51	53	52	52-53	15	0				
D. krusensternii	51-53	50-53	54	52	55-56	14	11	0			
D. elongata	55-56	52-56	55	52	56-57	22	18	19	0		
D. albobrunnea	54-62	52-56	57	53	57-58	20	16	19	16	0	
D. atromaculata	48-52	47-52	52	51	53-54	19	19	17	23	20	0

Table S3. Intra- and interspecific pairwise uncorrected p-distances of *Dendrodoris* based on CO1 data set. Ranges between minimum and maximum distances are given as percentages. Group B: *D. temarana* and *D. grandiflora* (only 2 sequences).

	D. nigra (clade c) Persian Gulf	*D. nigra* (clade a)	*D. nigra* (clade b)	Group B (Mediterranean sea)	*D. grandiflora*	*D. krebsii*	*D. senegalensis*	*D. herytra*	*D. limbata*	*D. fumata* (clade e)	*D. fumata* (clade f)	*D. rubra* (clade d)	*D. arborescens*	*D. guttata*	*D. citrina*	*D. krusensternii* (clade g)	*D. krusensternii* (clade h)	*D. krusensternii* (clade i)	*D. krusensternii* (clade j)	*D. krusensternii* (clade k)	*D. atromaculata*	*D. tuberculosa*
D. nigra (clade c Persian Gulf)	0-2																					
D. nigra (clade a)	11-12	0-2																				
D. nigra (clade b)	11-13	7-8	0																			
Group B (Mediterranean sea)	26-30	25-30	27-37	0-3																		
D. grandiflora	25-28	25-27	27-29	8-11	0-1																	
D. krebsii	24-25	23-24	26-29	13-17	12-14	0																
D. senegalensis	26-27	26-27	27-29	14-17	13-14	16	0-1															
D. herytra	27-28	28-29	31-33	16-20	17-18	19	20	0														
D. limbata	23-26	23-25	25-27	18-22	16-18	17-20	18-26	25-26	0-2													
D. fumata (clade e)	22-25	23-25	25-31	16-19	16-18	18-21	19-25	23-25	16-18	0-2												
D. fumata (clade f)	24-25	25-26	26-30	16-19	19-20	22	21-26	26	18-19	4-6	0											
D. rubra (clade d)	25-27	24-27	27-32	17-19	16-18	21-22	20-25	24-25	16-18	8-11	9-10	0-3										
D. arborenscens	26-28	27-28	27-31	18-21	17-18	20-21	19-24	24	17-18	18-19	20	20-21	0									
D. guttata	27-29	28-29	26-30	20-23	20-21	24	18-23	22-23	20	18-21	20	19-23	17-19	0-1								
D. citrina	51-58	49-51	52-59	52-59	46-51	51-52	51-57	57	44-47	47-48	48	48-49	48	51-52	0							
D. krusensternii (clade g)	60-66	60-61	56-69	63-70	59-62	62	66-67	67	56-57	60-62	62	63-64	59	61-62	20	0						
D. krusensternii (clade h)	52-60	54	52-55	55-65	50-54	55-56	56-59	57-67	51-54	53-55	57	57-58	56	58-59	19	8	0					
D. krusensternii (clade i)	59-67	57-58	55-58	59-68	58-61	61-62	62-64	59	58-59	58-61	60-61	60-64	58-59	60-62	22	8	6	0				
D. krusensternii (clade j)	59-62	56-58	55-70	65-69	61-63	64	65-68	63-64	60-61	62-63	63	63-65	58-59	64	20	8	6	7-8	0			
D. krusensternii (clade k)	55-63	56-57	56-61	53-64	50-54	57	57-61	61	53-55	54-56	57	57-58	54-55	59-60	20	11	8	11	13	0		
D. atromaculata	56-61	59-60	58-63	61-54	51-54	55-56	57-60	60	50-54	51-55	55	54-56	58-59	59	22	22	19	22	22	21	0	
D. tuberculosa	60-65	59-60	61-65	57-66	52-57	61-62	54-58	58	52-56	52-56	55	54-56	55-56	53-54	23	22	23	24	21	23	22	0

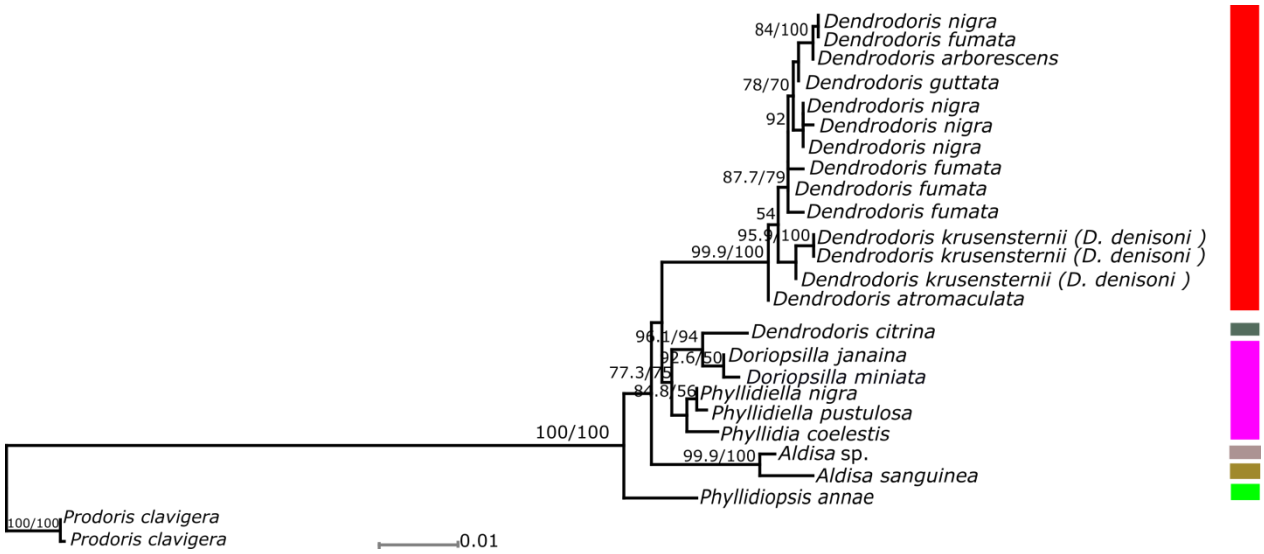

Fig. S1. Phylogenetic reconstruction of Dendrodorididae O'Donoghue, 1924 based on the 18S data set, with *Prodoris clavigera* (Thiele, 1912) as outgroup. Numbers before and after slash indicate approximate likelihood ratio test (SH-aLRT) and bootstrap values for maximum likelihood (ML) respectively. Numbers less than 50 are removed. Coloured bars indicate species delimitation resulting from ABGD test.

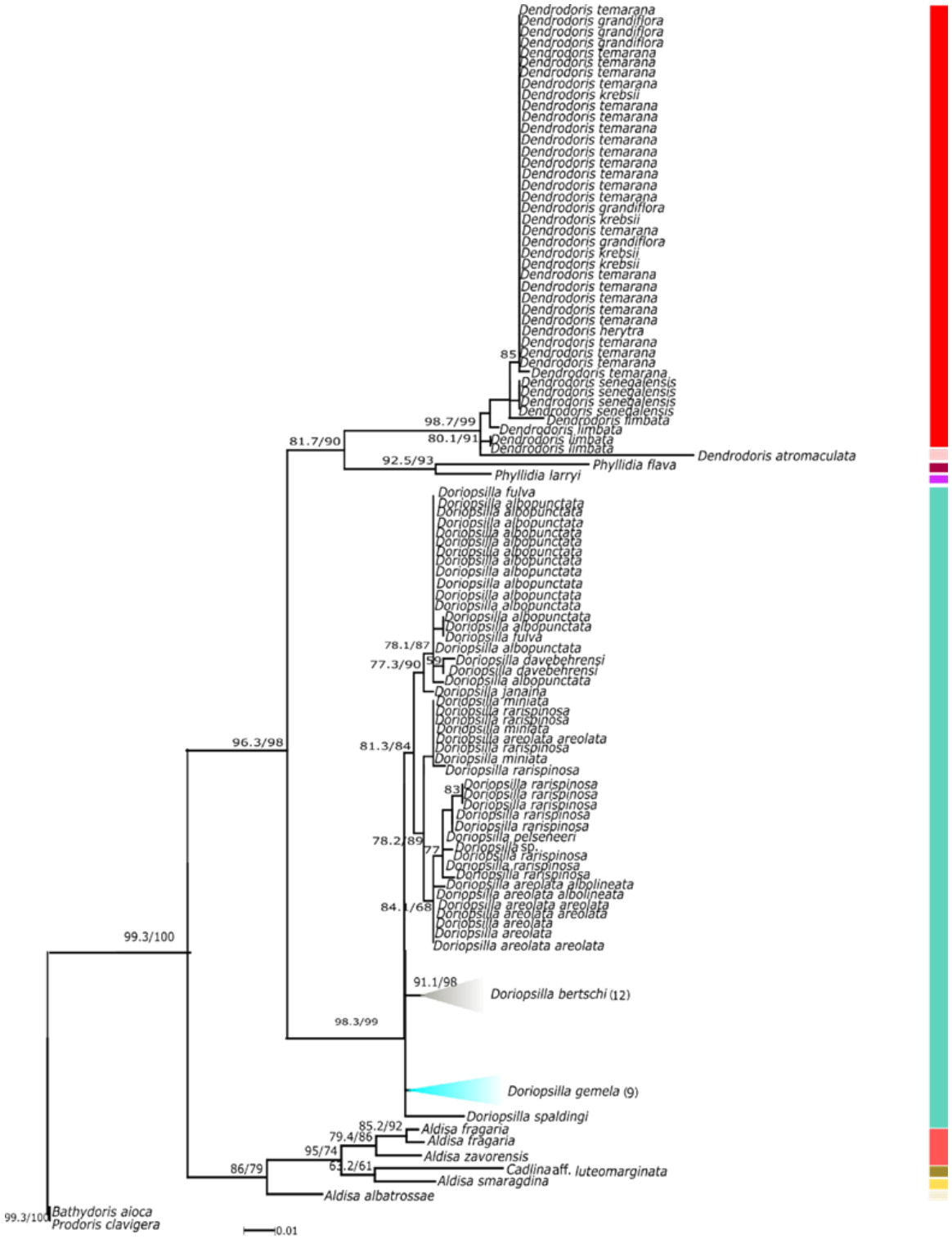

Fig. S2. Phylogenetic reconstruction of Dendrodorididae O'Donoghue, 1924 based on the H3 data set, with *Bathydoris aioca* Er. Marcus & Ev. Marcus, 1962 and *Prodoris clavigera* (Thiele, 1912) as outgroup. Terminal taxa on species level partly collapsed (triangles coloured for better clarity) and specimen numbers written in brackets. Numbers before and after slash indicate

approximate likelihood ratio test (SH-aLRT) and bootstrap values for maximum likelihood (ML) respectively. Numbers less than 50 are removed. Coloured bars indicate species delimitation resulting from ABGD test. Note that the genera *Dendrodoris* Ehrenberg, 1831 and *Doriopsilla* Bergh, 1880 are monophyletic.

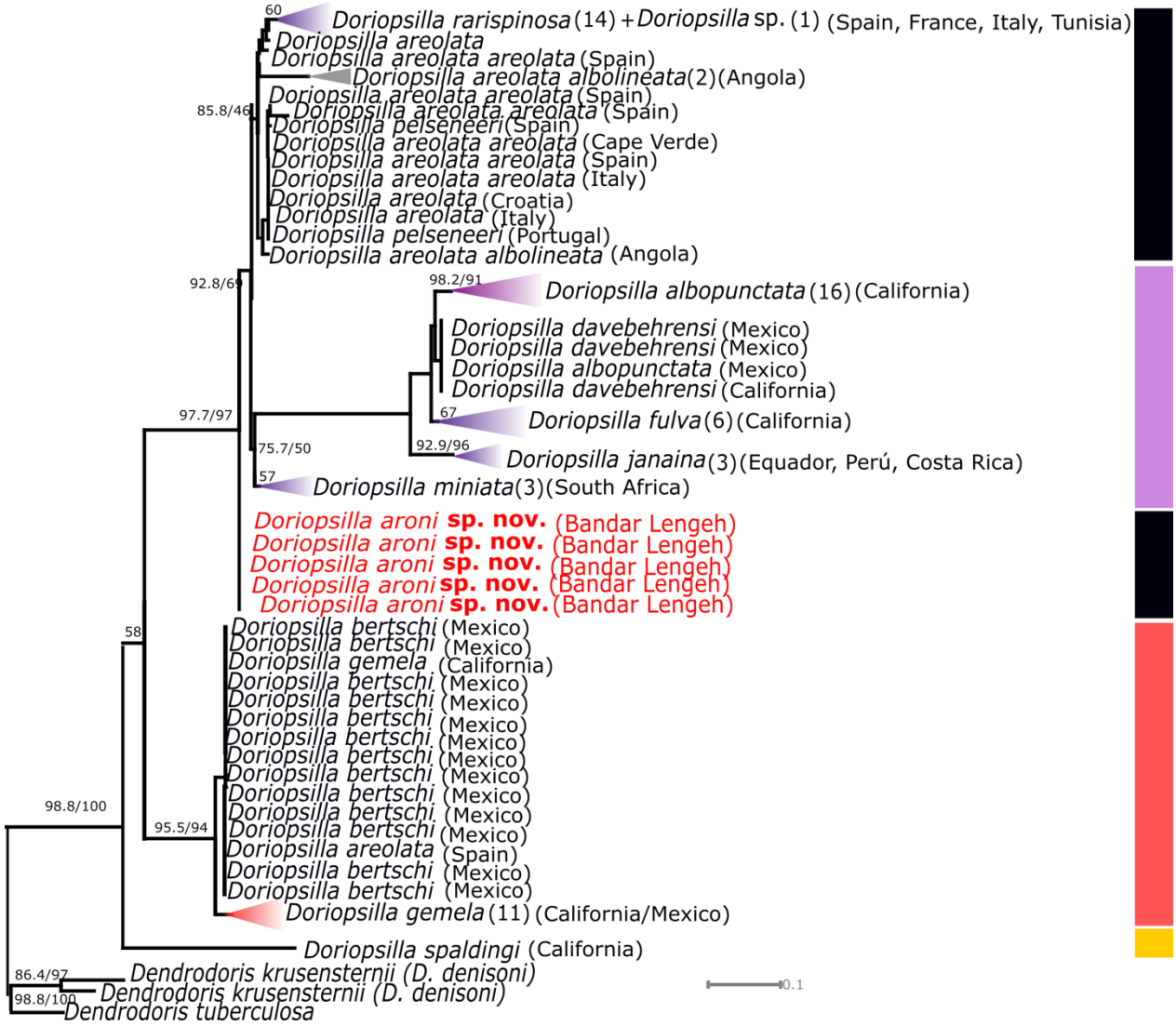

Fig. S3. Phylogenetic reconstruction of the genus *Doriopsilla* Bergh, 1880 based on the 16S data set, with *Dendrodoris* Ehrenberg, 1831 species (from short branch) as outgroup. Terminal taxa on species level partly collapsed (triangles coloured for better clarity) and specimen numbers written in brackets. Numbers before and after slash indicate approximate likelihood ratio test (SH-aLRT) and bootstrap values for maximum likelihood (ML) respectively. Numbers less than 50 are removed. Coloured bars indicate species delimitation resulting from ABGD test.

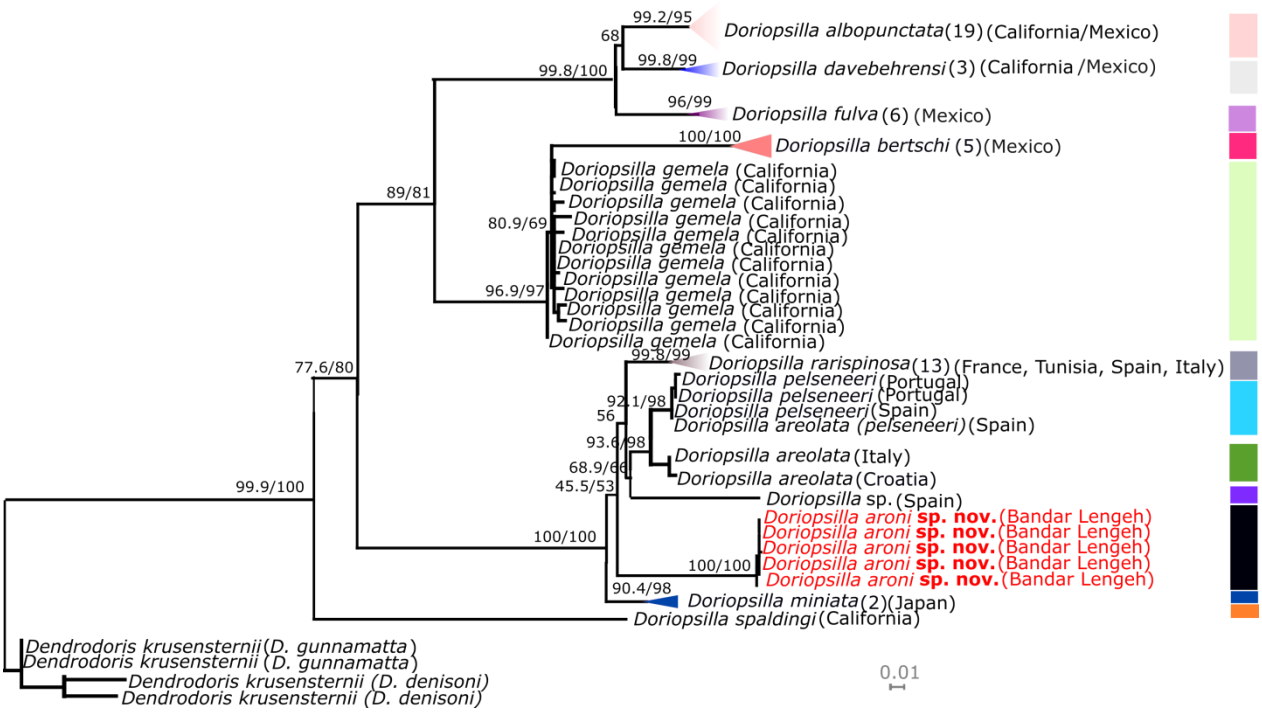

Fig. S4. Phylogenetic reconstruction of the genus *Doriopsilla* Bergh, 1880 based on the CO1 data set, with *Dendrodoris* Bergh, 1880 species (from short branch) as outgroups. Terminal taxa on species level partly collapsed (triangles coloured for better clarity) and specimen numbers written in brackets. Numbers before and after slash indicate approximate likelihood ratio test (SH-aLRT) and bootstrap values for maximum likelihood (ML) respectively. Numbers less than 50 are removed. Coloured bars indicate species delimitation resulting from ABGD test.

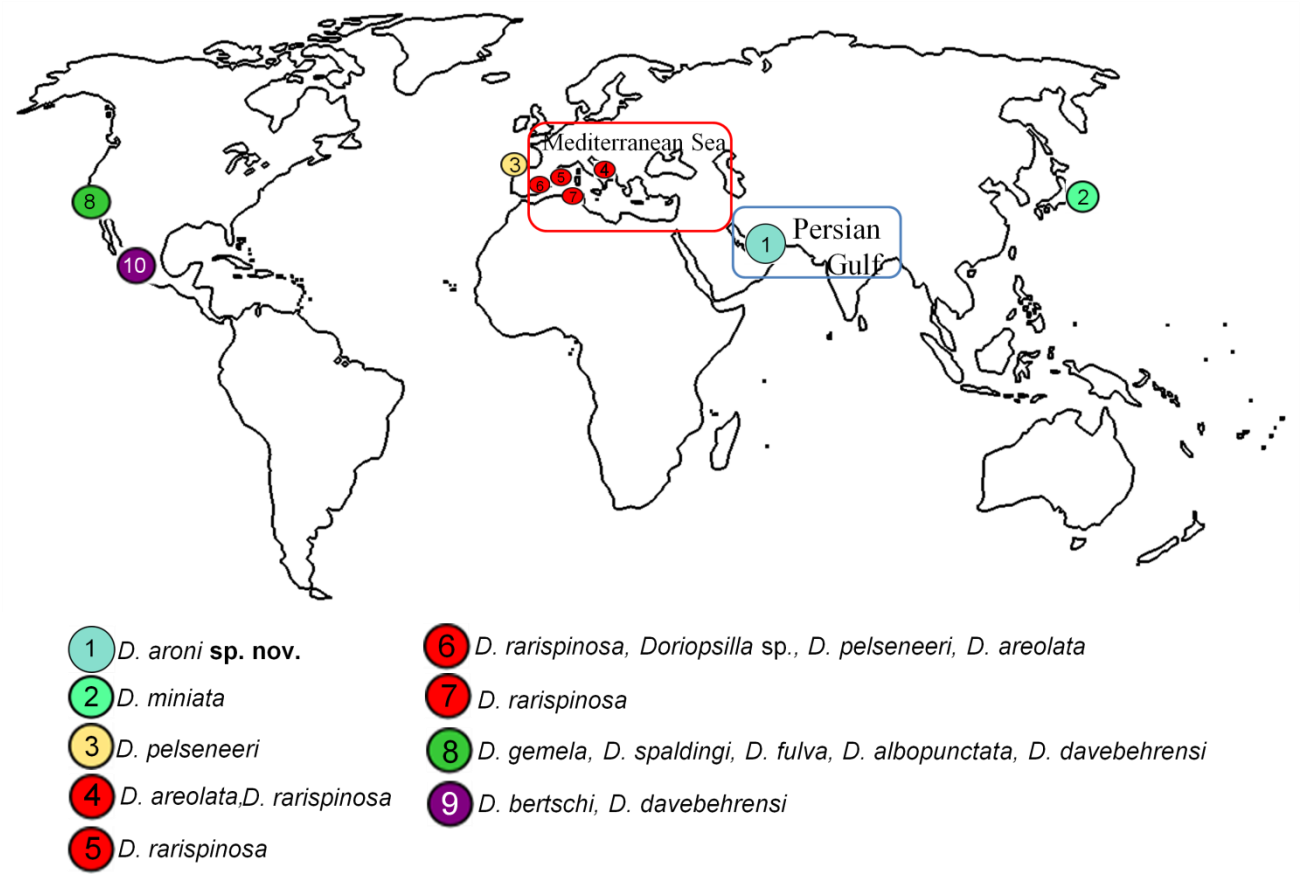

Fig. S5. Distribution map of *Doriopsilla* species (CO1 sequences) included in our study. Species and their respective localities highlighted in the same colour as in Fig.13. Outline world map downloaded from https://www.outline-world-map.com/blank-thick-white-world-map-b3c.

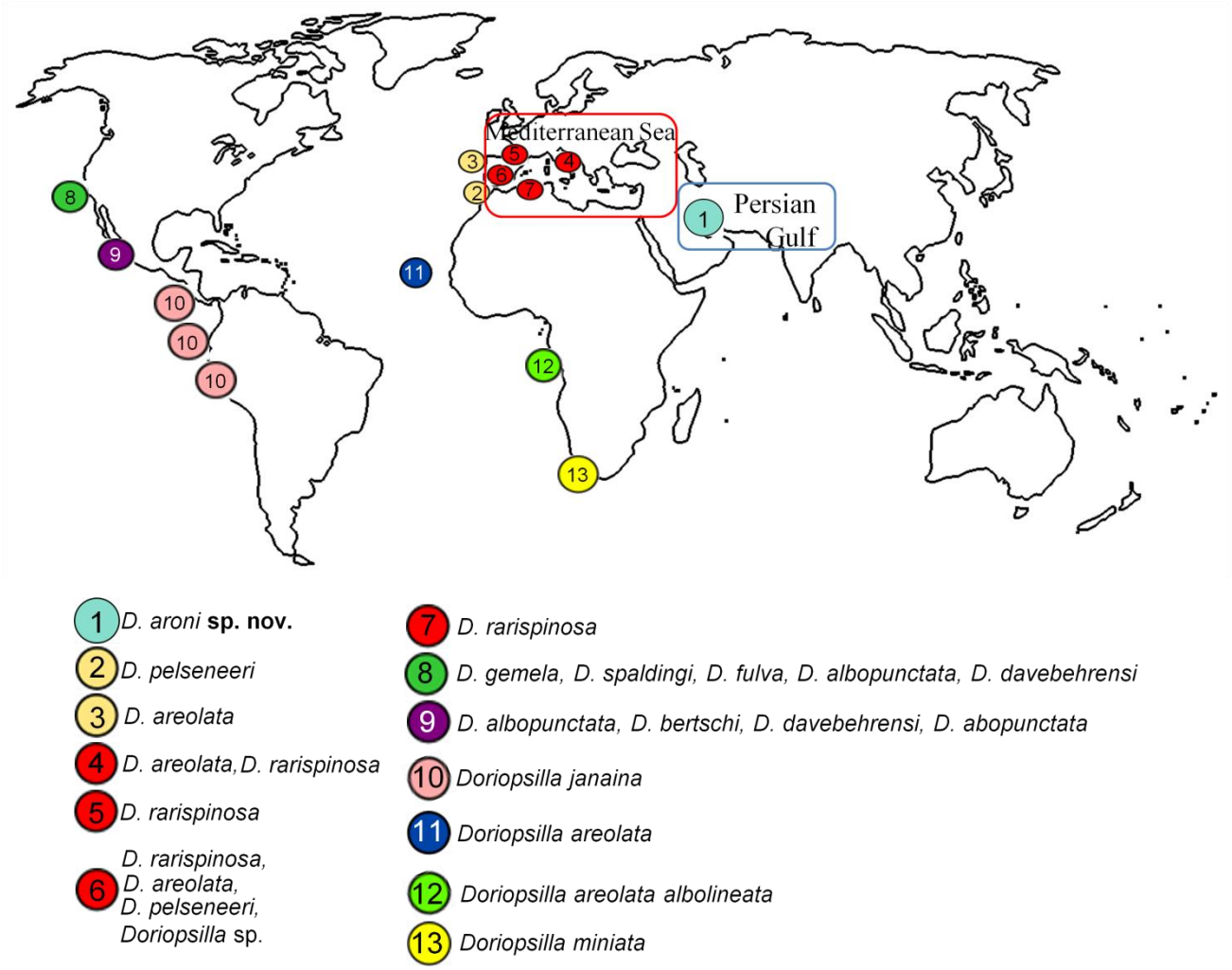

Fig. S6. Distribution map of *Doriopsilla* species (16S sequences) included in our study. Species and their respective localities highlighted in the same colour as in Fig. 14. Outline world map downloaded from https://www.outline-world-map.com/blank-thick-white-world-map-b3c.

Chapter 6

General Discussion

Onchidiidae

Morphology: the description and comparison of various species of the genus *Peronia*, focusing on a new species, *P. persiae* was disscussed. The distinguishing features of this new species are highlighted, including branchial gills covering only the posterior part of the body. This is in contrast to *P. peronii* with gills dispersed across the entire notum (Plate 1893; Labbé 1934; Solem 1959). The absence of spicules in the notum is another distinguishing feature of this new species, along with a strongly pigmented visceral cavity. This contrasts with *P. peronii*, which lacks pigmentation in the visceral cavity (Labbé 1934; Plate 1893). The new species possesses specific photoreceptor structures and the intestinal type and the position of the nephridium are also mentioned as distinguishing features. Furthermore, the structure of the genital systems and penial complex in *P. persiae* are compared to those of other *Peronia* species. For instance, *P. verruculata* differs by fork-shaped and short spines located in the posterior part of the penis (Awati and Karandikar 1948; Britton 1984). Based on these anatomical differences supported by molecular data and metabolomics investigation, we propose *P. persiae* as a distinct new species.

Molecular data: The classification of the genus *Peronia* has posed such a formidable challenge that it has been largely avoided for many decades. Labbé (1934) stands as the most recent author to have assigned species names to onchidiids featuring dorsal gills, with the exception of the recent addition, *P. persiae* (see Dayrat *et al*., 2020). We present the first molecular analysis of the all Onchidiidae genera, including the genus *Peronia*. The analysis shows evidence for the monophyly of *Peronia* and most other onchidiid genera, while some genera like *Platevindex* and *Onchidium* are not monophyletic. Recently, Goulding *et al*., (2021) revised the taxonomy of *Platevindex,* including acknowledged species and a new species named *Platevindex aptei* Goulding & Dayrat, 2021. The monophyly of *Platevindex* was confirmed through molecular and anatomical studies. Similarly, the monophyly of *Peronia* was also supported. In the present study, species delimitation tests confirm the distinctness of *P. persiae* from other *Peronia* species based on molecular data. The analysis also indicates the presence of several other, possibly undescribed, *Peronia* species. Simultaneously, Dayrat *et al.*, (2020) revised the genus *Peronia*.

They identified 31 group names within the *Peronia* species, deeming 27 of these as invalid and recognizing four as valid. Furthermore, they introduced five new species: *P. Griffithsi* Dayrat & Goulding, 2020, *P. okinawensis* Dayrat & Goulding, 2020, *P. setoensis* Dayrat & Goulding, 2020, *P. sydneyensis* Dayrat & Goulding, 2020, and *P. willani* Dayrat & Goulding, 2020. The genetic distance analysis separates specimens from Singapore into two groups, indicating the possible presence of an undescribed cryptic species within the genus. Goulding *et al*., (2022) provided a haplotype network of approximately 170 CO1 sequences of *P. verruculata* between the western and northern Indian Ocean and the central Indo-West Pacific, including the *P. veruculata* used in present study. In general, he concluded that divergence in specimens could reflect genetic distances between populations or could be a result of limited dispersal by currents, as we also observed in clade 4 of our haplotype network.

Metabolomic investigations: We discussed the chemical distinctiveness of *P. persiae* and *P. verruculata* through metabolomic investigations. Both species show a diverse mixture of polypropionate esters, which are common compounds in Onchidiidae. However, the two species differ in the types and ratios of these compounds. *P. persiae* contains flavonoids genistein and daidzein as common secondary metabolites, while cholesterol and bile acids are identified exclusively in *P. verruculata*. Both species also share hydrophilic zwitterionic amino acid-betaine compounds, which are important osmolytes in response to hyperosmotic conditions. This study suggests that these metabolites may be involved in chemical defense and coping with osmotic stress. Arast *et al*., 2022 investigated toxicity of *P. peronii* through MTT assay and apoptosis assay on melanoma tumor mitochondria. Animals were native from Bushehr province located on the northern shores of the Persian Gulf. They showed metabolites in *P. peronii* extract, with antimicrobial properties, target mitochondria in cancer cells. Jahani *et al*., 2019 studied on *P. veruuculata* again sampled from Bushehr Province and showed that the extract of this marine animal had an antibacterial effect. We cannot confirm that the studied samples are not *P. persiae* because molecular analysis was not performed, but in general it is proved that the genus *Peronia* from the northern Persian Gulf plays an important role in drug discovery and further studies are needed.

Dendrodorididae

Molecular

***Dendrodoris*:** In the study by Hallas *et al*., (2017), mitochondrial gene analysis revealed a significant genetic divergence among certain *Dendrodoris* species, including *D. nigra*, *D. fumata*, *D. arborescens*, and *D. guttata*, separating them from other *Dendrodoris* species. The study presented here also identified an additional six species within this divergent group, characterized by an unusually large genetic distance of more than 70% compared to other related species within the Dendrodorididae, Phyllidiidae, and dorid species. This divergence might be attributed to introgression, but further investigation is needed. Additionally, the analysis of nuclear genes supported the monophyly of *Dendrodoris*.

Regarding *D. nigra* and *D. fumata*, there has been historical taxonomic confusion, with some considering *D. nigra* as a junior synonym of *D. fumata* (Gohar & Soliman 1967; Hirose *et al*., 2014; Brodie *et al*., 1997; Valdés & Gosliner 1999; Yonow 2012; Tibiriçá *et al*., 2017; Gosliner *et al*., 2018). However, molecular analysis, particularly of mitochondrial genes, confirms that *D. nigra* and *D. fumata* are indeed distinct species. Notably, mitochondrial gene 16S appeared less variable than CO1, which was preferred for species delimitation. The study identified multiple clades within *D. nigra*, indicating potential cryptic variation and the need for further examination.

Furthermore, there has been uncertainty about the taxonomic status of *D. fumata* and *D. rubra* (Gohar & Soliman 1967; Brodie *et al*., 1997). While morphological studies suggested *D. rubra* as a junior synonym of *D. fumata* (Hirose *et al*., 2014; Fatemi *et al*., 2021). Our molecular analyses, based on CO1 analyses, support the distinction of *D. fumata* and *D. rubra* as separate species. Additionally, sequences from Hawaii were assigned to *D. rubra*.

***Doriopsilla*:** In terms of the systematics of *Doriopsilla* species and species complexes, studies have been relatively limited. Furfaro *et al*., (2022) recently addressed the *D. areolata* species complex along the Mediterranean and south-east Atlantic coastline using CO1 sequences, identifying four distinct species: *D. areolata*, *D. rarispinosa* (Mediterranean Sea), *D. pelseneeri*, and another likely undescribed species (Mediterranean and Atlantic coast of Spain). Hoover *et al*., (2015) clarified the situation of north-eastern Pacific species, resurrecting *D. fulva* and describing two new species. These studies contradicted previous analyses based on 16S

mitochondrial gene sequences, which led to the synonymization of the *D. areolata* species complex with *D. miniata* (Goodheart & Valdés 2013).

The species delimitation analyses conducted in this study, which utilized the 16S dataset, suggested that 16S sequences are conservative when it comes to species discrimination in Dendrodorididae. As a result, the analyses using the CO1 dataset are deemed more relevant. In these analyses, specimens from the Persian Gulf were identified as a distinct species more closely related to species from the Mediterranean or the eastern Atlantic. *D. miniata* also showed a closer relationship, despite the significant geographical distance between their locations (west coast of South Africa and Japan compared to the Persian Gulf) (Goodheart & Valdés 2013; Gosliner *et al*., 2008; GBIF). However, no sequences from the type locality of *D. miniata* in the south-east coast of India are available. This new species is similar in color to *D. miniata* but lacks the characteristic network of white lines on the notum present in *D. miniata*.

The sequence divergence between the new species and the closest related species (CO1: 10-14%) falls within or exceeds the range observed between valid species. The lowest values were found to be 5-7% between *D. areolata*, *D. pelseneeri*, and *D. rarispinosa* from the Mediterranean/Atlantic region, and 6-7% between *D. miniata* specimens from Japan and *D. rarispinosa* from the Mediterranean region. Notably, *D. nigrocera*, which has been recorded exclusively from the Iranian coast, is characterized by distinctive black rhinophores, a feature absent in the new species and any other described species (Yonow, 2012). Specimens mentioned by Nithyanandan (2012) from the south coast of the Persian Gulf (Kuwait) under the name *Doriopsilla cf. miniata* also likely belong to this new species due to the absence of the reticulated white lines characteristic of *D. miniata*.

Morphology

***Dendrodoris*:** Previous descriptions of *D. nigra* and *D. fumata* were primarily based on material from Australia and Hawaii, with limited histological information available (Wägele *et al*., 1999). Molecular analyses have revealed cryptic variations in these species, prompting a comparative analysis of their morphology and histology.

For *D. nigra*, specimens from the Persian Gulf exhibit distinct characteristics not previously documented in descriptions from Australia and Hawaii. These include the presence of reddish-stained glandular cells surrounding the oral tube, a sac-like stomach (in contrast to an

undifferentiated stomach previously reported), and the presence of spines on the penis. Notably, the absence of a cuticle on the penis in the Persian Gulf specimens raises questions about the variability of penial spines in *Dendrodoris* species. Furthermore, we highlight the presence of well-developed nidamental glands and a large vestibular gland in a relatively small 19 mm specimen, indicating full maturity, in contrast to larger specimens from previous studies that lacked these developments. These morphological findings align with molecular data, suggesting a separate lineage in Persian Gulf specimens compared to those mainly from the Pacific Ocean.

The presence or absence of penial hooks in different color morphs of *D. fumata*, including the gray and red forms with spines and the black form lacking the spines, has been confirmed by previous researchers (Brodie *et al*., 1997; Ev. & Er. Marcus 1970; Edmunds 1971; Gohar & Soliman 1967; Eliot 1904). We highlight that Brodie (2001) provided some histological results for this species, mainly focused on the digestive tract, and described the presence of the vestibular gland for the first time in *D. fumata*, which is confirmed in the current study.

Another interesting observation was a thick cuticle on the penis of the investigated specimen, which has a length of 23 mm. It suggests that future studies combining molecular data with information on color, specimen size, and the presence or absence of penial spines may reveal ontogenetic differences or hidden variations not previously identified in *D. fumata*.

Additionally, we emphasis the presence of symbiotic bacteria within the epithelium of the vestibular gland in *D. nigra*, a unique character previously described in the literatures (Klussmann-Kolb and Brodie 1999; Wägele *et al*., 1999). This characteristic is observed in both *D. nigra* and *D. fumata* specimens from the investigation, expanding our understanding of these species.

***Doriopsilla*:** We analyses various *Doriopsilla* species via DNA barcoding reported from different regions, primarily focusing on the South Australia region and the Atlantic coast of southern Africa. It notes distinct characteristics of these species, such as coloration, size, and anatomical features, which differentiate them from *Doriopsilla aroni* **sp. nov.**. Notably, *D. aroni* **sp. nov.** lacks the white spots observed in *D. aurea* and the granules on the notum seen in *D. carneola*. Moreover, *D. peculiaris*, *D. debruini*, and *D. capensis* have unique coloration and anatomical features that distinguish them from the new species.

A close relative to *D. aroni* **sp. nov.** is *D. miniata*, known for its varying shades of orange and characteristic white lines. Despite the similarities, our study differentiates *D. aroni* **sp. nov.** by its absence of white lines on the notum. Comparisons are also made with Mediterranean species, *D. areolata* and *D. rarispinosa*, which exhibit a typical white network on the notum, unlike *D. aroni* **sp. nov.**. The anatomical features, particularly penial armature, can be a potential way of distinguishing between these species, as differences in spine size and arrangement are noted. It also discusses the histological aspects of *D. aroni* **sp. nov.,** emphasizing its reproductive maturity based on the receptaculum seminis and prostate gland contents.

Chapter 7

General Conclusions

In conclusion, this study examined the morphology and molecular data of various species within the genera *Peronia*, *Doriopsilla*, and *Dendrodoris*. It introduced a new species, *P. persiae*, in the *Peronia* genus and a new species, *D. aroni* **sp. nov.**, in the *Doriopsilla* genus. Key characteristics of these new species were emphasized and compared to others within their respective genera.

The molecular analysis provided strong support for the monophyly of *Peronia* and most other onchidiid genera. Species delimitation tests affirmed the uniqueness of *P. persiae* among other *Peronia* species based on molecular data. Additionally, metabolomic investigations revealed chemical distinctions between *P. persiae* and *P. verruculata*, suggesting a diverse mixture of polypropionate esters and variations in compound types and ratios. These metabolites may play roles in chemical defense and osmotic stress adaptation.

Furthermore, new *Doriopsilla* specimens from the Iranian coastline were identified as a distinct species not matching any known *Doriopsilla* species. The study confirmed the presence of at least two *Dendrodoris* species, *D. nigra* and *D. fumata*. It also suggested that the CO1 marker is more suitable for biodiversity studies within the Dendrodorididae family. However, questions remain about the cryptic diversification of *D. nigra*, with histological investigations potentially supporting molecular findings. Lastly, our study emphasized the need to understand why mitochondrial genes have caused significant genetic divergence in a group of *Dendrodoris* species, possibly requiring mitochondrial and nuclear genomic analyses for clarification.

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