Supplementary Information
for the article Multilevel fine-scale diversity challenges the 'cryptic species' concept

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Text (Materials and Methods)

Sample data

Material for this study was obtained by scuba diving at widely separated locations in Europe. Specimens were collected in Norway (west coast, near the mouth of the Sogne fjord, Gulen Dive Resort), Sweden (west coast, Bohuslän county, Smögen Dive Resort), Ireland (Killary Harbour), United Kingdom (Northern Ireland, Strangford Lough), Spain (L'Estartit, Girona), Italy, France, Croatia and in the Black Sea, Russia (Yalta region). The specimens were preserved in 95–96% or 70% ethanol and deposited in the Zoological Museum of Lomonosov Moscow State University (ZMMU), in the Gothenburg Natural History Museum (GNM) and National Museums Northern Ireland (BELUM.Mn), and Department of Science of the Roma Tre University (RM3).

Morphological analysis

The morphology of the nudibranchs and their egg masses was studied under a stereomicroscope and using Nikon D810 and Nikon D600 digital cameras. For the description of internal features both preserved and fresh specimens (when available) were dissected under the stereomicroscope. The buccal mass of each specimen was extracted and processed in 10% sodium hypochlorite solution. The features of the jaws of each species were analysed under the stereomicroscope and scanning electron microscope and were then drawn. The coated radulae were examined and photographed using a scanning electron microscope (CamScan, JSM). The reproductive systems were also examined and drawn using the stereomicroscope.

Molecular analysis

A total of 17 specimens were successfully sequenced for the mitochondrial genes cytochrome c oxidase subunit I (COI), 16S rRNA, and the nuclear genes Histone 3 (H3) (see Table for the primers). Of these, one specimen was sequenced in the United Kingdom, three specimens were sequenced in Rome, the others in Moscow. Additional molecular data for 12 specimens of nudibranchs were obtained from GenBank (see Table S1). DNA extraction procedure, PCR amplification options, and sequence obtainment is previously described in detail (Korshunova, T.A. *et al.* 2017a, 2017b,2018; Morrow *et al.* 2012, Furfaro 2016 *et al.*). Protein coding sequences were translated into amino acids for confirmation of the alignment. All sequences were deposited in GenBank (see Supporting Information, Table S1, highlighted in bold). Original data and publicly available sequences were aligned with the MAFFT algorithm (Katoh *et al.*2002). Separate analyses were conducted for COI (658 bp), 16S (442 bp), H3 (328 bp) and concatenated data (1428 bp). Evolutionary models for each data set were selected using MrModelTest 2.3 under the Akaike information criterion (Nylander et al. 2004 ,Akaike 1974). Two different phylogenetic methods, Bayesian inference (BI) and Maximum Likelihood (ML), were used to infer evolutionary relationships. Bayesian estimation of posterior probability was performed in MrBayes 3.2 (Ronquist et al. 2012). Four Markov chains were sampled at intervals of 500 generations. Analysis was started with random starting trees and 10^7 generations. ML analysis was performed using RAxML 7.2.8 (Stamatakis et al. 2008) with 1000 bootstrap replicates. Final phylogenetic tree images were rendered in FigTree 1.4.2. To evaluate the genetic distribution of the different haplotypes, a haplotype network for the COI molecular data was reconstructed using Population Analysis with Reticulate Trees (PopART, http://popart.otago.ac.nz) with the TCS network method. The program Mega7 (Kumar et al. 2016) was used to calculate the minimum uncorrected *p*-distances between all the sequences. Intra- and intergroup genetic distances were also examined. Additionally, Automatic Barcode Gap Discovery (ABGD) was used to define species (Puillandre et al. 2011). Alignment from the COI marker for *Trinchesia* specimens were submitted and processed in ABGD using the Jukes-Cantor (JC69) and Kimura (K80) models and the following settings: a prior for the maximum value of intraspecific divergence between 0.001 and 0.1, 20 recursive steps.

Primers used for amplification.

Name	5′→3′	References
LCO 1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al*., 1994
HCO 2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al*., 1994
16S arL	CGCCTGTTTAACAAAAACAT	Palumbi *et al*. 2002
16S R	CCGRTYTGAACTCAGCTCACG	Puslednik & Serb, 2008
H3 AF	ATGGCTCGTACCAAGCAGACGG	Colgan *et al*., 1998
H3 AR	ATATCCTTGGGCATGATGGTGAC	Colgan *et al*., 1998

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Tables S1–S5

Table S1. List of specimens used for molecular analyses.

Species	Locality	Registration	GenBank accession nos.		
			COI	16S	H3
Diaphoreolis lagunae (O'Donoghue, 1926)	California	CAS179465a	KY128956	KY128749	KY128543
Diaphoreolis viridis (Forbes, 1840)	Russia, White Sea	ZMMU:Op-537	MG266028	MG266026	MG266029
Trinchesia diljuvia **sp.n.**	Russia, Black Sea	ZMMU:Op-642	**MK587917**	**MK587933**	**MK587903**
Trinchesia diljuvia **sp.n.**	Russia, Black Sea	ZMMU:Op-643	**MK587918**	**MK587934**	**MK587904**
Trinchesia caerulea (Montagu, 1804)	Norway, Gulen	ZMMU:Op-622	MG266024	MG266022	MG266025
Trinchesia caerulea (Montagu, 1804)	North Sea	-	AF249807	-	-
Trinchesia caerulea (Montagu, 1804)	Ireland	ZMMU:Op-646	**MK587928**	**MK587930**	-
Trinchesia caerulea (Montagu, 1804)	Spain, L'Estartit, Girona	ZMMU:Op-648	**MK587915**	**MK587931**	**MK587898**
Trinchesia caerulea (Montagu, 1804)	Spain, L'Estartit, Girona	ZMMU:Op-649	**MK587916**	**MK587932**	**MK587899**
Trinchesia caerulea (Montagu, 1804)	Italy, Tor Paterno	RM3_309	**MK587912**	-	**MK587896**
Trinchesia caerulea (Montagu, 1804)	Italy, Tor Paterno	RM3_333	**MK587913**	**MK587929**	**MK587897**
Trinchesia caerulea (Montagu, 1804)	Croatia, Ugljan Island	ZMMU Op-647	**MK587914**	-	-
Trinchesia cuanensis **sp.n.**	United Kingdom	GNM 9099	KY128911	KY128703	KY128498
Trinchesia cuanensis **sp.n.**	United Kingdom	ZMMU: Op-650	**MK587922**	MK587936	MK587906
Trinchesia cuanensis **sp.n.**	United Kingdom	GNM 9054	**MK587920**	**MK587935**	**MK587905**
Trinchesia cuanensis **sp.n.**	United Kingdom	BELUM.Mn.2018.2	**MK587919**	**-**	**-**
Trinchesia cuanensis **sp.n.**	North Sea, Sweden	GNM 9243	**MK587921**	**-**	**-**
Trinchesia lenkae Martynov, 2002	Japan: Hokkaido	ZMMU:Op-619	MG242334	MG242333	**MK587900**
Trinchesia lenkae Martynov, 2002	Japan: Hokkaido	ZMMU:Op-620	MG242336	MG242335	**MK587901**
Trinchesia lenkae Martynov, 2002	Russia: Sea of Japan	ZMMU:Op-621	MG242338	MG242337	**MK587902**
Trinchesia morrowae **sp.n.**	France, Banyuls-sur-Mer	ZMMU:Op-535	**MK587923**	**MK587937**	**MK587907**
Trinchesia morrowae **sp.n.**	France, Banyuls-sur-Mer	ZMMU:Op-653	**MK587926**	**MK587940**	**MK587910**

Trinchesia morrowae **sp.n.**	France, Banyuls-sur-Mer	ZMMU Op-656	**MK587927**	**MK587941**	**MK587911**
Trinchesia morrowae **sp.n.**	Spain, L'Estartit, Girona	ZMMU:Op-651	**MK587924**	**MK587938**	**MK587908**
Trinchesia morrowae **sp.n.**	Spain, L'Estartit, Girona	ZMMU Op-652	**MK587925**	**MK587939**	**MK587909**
Trinchesia morrowae **sp.n.**	Spain	CAS185199	KY128922	KY128717	KY128510
Trinchesia ornata (Baba, 1937)	Hawaii	CAS180344	KY128967	KY128758	KY128553
Trinchesia sibogae (Bergh, 1905)	Philippines	CAS177489	KY128975	KY128767	KY128562
Trinchesia speciosa (Macnae, 1954)	South Africa	CAS176913	KY128996	KY128788	KY128583
Trinchesia speciosa (Macnae, 1954)	South Africa	CAS176954	KY128998	KY128790	KY128585

Table S2. Maximum intragroup (highlighted in bold) and minimum intergroup genetic distances (%) for COI marker in *Trinchesia* species.

	T. caerulea	*T. cuanensis* **sp.n.**	*T. morrowae* **sp.n.**	*T. diljuvia* **sp.n.**	*T. lenkae*	*T. speciosa*	*T. ornata*	*T. sibogae*
T.caerulea	**1.25**	7.20	10.95	11.89	16.74	15.18	17.45	17.06
T. cuanensis **sp.n.**	7.20	**1.88**	11.27	12.99	18.5	16.43	17.45	17.53
T. morrowae **sp.n.**	10.95	11.27	**2.18**	2.98	17.84	14.71	15.88	16.12
T. diljuvia **sp.n.**	11.89	12.99	2.98	**0.16**	17.37	15.96	16.67	17.53
T. lenkae	16.74	18.5	17.84	17.37	**0.31**	16.12	13.36	18.94
T. speciosa	15.18	16.43	14.71	15.96	16.12	**0.78**	14.62	16.28
T. ornata	17.45	17.45	15.88	16.67	13.36	14.62	-	17.14
T. sibogae	17.06	17.53	16.12	17.53	18.94	16.28	17.14	-

Table S3. Maximum intragroup (highlighted in bold) and minimum intergroup genetic distances (%) for 16S marker in *Trinchesia* species.

	T. caerulea	*T. cuanensis* **sp.n.**	*T. morrowae* **sp.n.**	*T. diljuvia* **sp.n.**	*T. lenkae*	*T. speciosa*	*T. ornata*	*T. sibogae*
T.caerulea	**0.25**	1.39	5.31	6.47	11.52	11.52	10.54	11.03
T. cuanensis **sp.n.**	1.39	**0.46**	6.0	6.24	12.01	11.55	11.32	10.62
T. morrowae **sp.n.**	5.31	6.0	**0.23**	1.62	11.32	10.39	11.55	10.39
T. diljuvia **sp.n.**	6.47	6.24	1.62	**0.46**	11.55	10.39	11.78	10.62
T. lenkae	11.52	12.01	11.32	11.55	**0.46**	8.78	4.39	10.39
T. speciosa	11.52	11.55	10.39	10.39	8.78	**0**	8.78	12.01
T. ornata	10.54	11.32	11.55	11.78	4.39	8.78	-	11.09
T. sibogae	11.03	10.62	10.39	10.62	10.39	12.01	11.09	-

Table S4. Maximum intragroup (highlighted in bold) and minimum intergroup genetic distances (%) for H3 marker in *Trinchesia* species.

	T. caerulea	*T. cuanensis* **sp.n.**	*T. morrowae* **sp.n.**	*T. diljuvia* **sp.n.**	*T. lenkae*	*T. speciosa*	*T. ornata*	*T. sibogae*
T.caerulea	**0.66**	0.98	1.63	1.63	4.25	5.88	4.9	3.27
T. cuanensis **sp.n.**	0.98	**0**	1.63	1.31	4.58	6.21	5.23	3.59
T. morrowae **sp.n.**	1.63	1.63	**0.65**	0.98	4.58	6.21	5.23	4.58
T. diljuvia **sp.n.**	1.63	1.31	0.98	**0**	4.58	6.21	5.23	4.25
T. lenkae	4.25	4.58	4.58	4.58	**0**	2.61	2.29	4.90
T. speciosa	5.88	6.21	6.21	6.21	2.61	**0**	3.59	7.52
T. ornata	4.9	5.23	5.23	5.23	2.29	3.59	-	5.56
T. sibogae	3.27	3.59	4.58	4.25	4.90	7.52	5.56	-

Table S5. Morphological characters that allow species of the *Trinchesia caerulea* complex to be distinguished.

	Adult length (live, max)	**Anterior ceratal rows**	**Anterior foot structures**	**White line across dorsum**	**Colour zones of cerata**	**Colour of digestive gland in ceratal bases**	**Lower orange band on cerata**	**Upper narrow black band on cerata**	**Radula**	**Receptaculum seminis**	**Stylet of the copulative organ**
T. caerulea	21 mm	Commonly 4	Long foot corners	Absent	Present	Greenish to light grayish	Weak or reduced	Absent	57-63 x 0.1.0	Oval, large, 8-shaped, but without lateral additional chamber	Short
T. cuanensis **sp.n.**	15 mm	Commonly 4	Short foot corners	Absent	Present	Blackish to dark grayish	Weak or reduced	Present	64 x 0.1.0	Rounded, large, with long convoluted additional chamber	Very long
T. morrowae **sp.n.**	10 mm	Commonly 3, rarely 4	Angular projections	Present	Present	Grayish to yellowish	Distinct, often broad	Absent	55-57x 0.1.0	Oval, small without lateral additional chamber	Short
T. diljuvia **sp.n.**	4.5 mm	Commonly 2, rarely 3	Distinct projections absent	Present	Absent	No distinct colour zones, dark grayish to light brownish or light greenish and covered with white or sometimes with light bluish dispersed pigment	Absent	Absent	27–34 x 0.1.0	Elongated, without lateral additional chamber	Relatively short