



Molecular data reveal cryptic lineages within the northeastern Atlantic and Mediterranean small mussel drills of the *Ocenebrina edwardsii* complex (Mollusca: Gastropoda: Muricidae)

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We used a molecular phylogenetic approach to investigate species delimitations and diversification in the mussel drills of the *Ocenebrina edwardsii* complex by means of a combination of nuclear (internal transcribed spacer 2, ITS2) and mitochondrial [cytochrome oxidase subunit I (COI) and 16S] sequences. Our sample included 243 specimens ascribed to seven currently accepted species from 51 sites. Five of the samples were from either the type locality of a nominal species or a close nearby locality (*O. edwardsii* from Corsica, *O. carmelae* and *O. piantonii* from the Kerkennah Islands, *O. hispidula* from the Gulf of Gabès and *O. leukos* from the Canary Islands), one from the inferred original locality (*O. ingloria* from Venice Lagoon), and specimens assigned in the recent literature to *O. nicolai*. We used a combination of distance- and tree-based species delimitation methods to identify Molecular Operational Taxonomic Units (MOTUs) to compare with the *a priori* species identifications. The consensus tree obtained by BEAST on the COI alignment allows the recognition of several distinct clades supported by the three species delimitation methods employed. The eight-MOTUs scenario, shared by the Automatic Barcode Gap Discovery (ABGD) and Generalized Mixed Yule-Coalescent (GMYC) methods, comprises the following major clades: clade A contains the south Tunisian species *Ocenebrina piantonii* Cecalupo, Buzzurro & Mariani from which the sympatric taxon *O. carmelae* Cecalupo, Buzzurro & Mariani (new synonym) cannot be separated; clades B and C bring together all populations from the Aegean Sea and some from the Ionian Sea, respectively; clade D groups, on the one hand, the south Tunisian samples morphologically assigned to *O. hispidula* Pally and, on the other, Atlantic and Alboran Sea samples (including the Canarian taxon *O. leukos* Houart); clade E includes a sample from the type locality of *O. edwardsii* and several samples from the Tyrrhenian Sea; clades F and G correspond to a few samples from the Venice Lagoon and the Tyrrhenian Sea, respectively; clade H groups the bulk of samples from the Adriatic Sea, including samples from the Venice Lagoon morphologically identified as *Ocenebrina ingloria* (Crosse), and some from the Ionian Sea. No final conclusions could be reached to reconcile the currently recognized morphological taxa with the clades suggested by the COI data. The geographical structure proposed by the mitochondrial markers is similar to that found in other marine invertebrates and partially corresponds to the species defined by shell characters. We propose here a framework for the revision of the *Ocenebrina edwardsii* species complex, suggesting a geographical pattern for the diversification of this group in the studied area.

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INTRODUCTION

The existence of sibling species among marine invertebrates is a widely recognized phenomenon (Knowlton, 1993), emphasized by the increasing use of DNA-based methods for species delimitation (Vogler & Monaghan, 2007). These techniques are contributing to the steady number of new marine species described (Costello, Wilson & Houlding, 2012), allowing the recognition of unknown diversity (Caron *et al.*, 2012) and testing the status of known species (Templeton, 2001).

Cryptic diversity, however, does not surface exclusively in poorly known lineages (Richards *et al.*, 2012) or in organisms from relatively unexplored areas (Goetze, 2003). Even within the well-known fauna of the Mediterranean Sea, with its long-lasting tradition of taxonomic studies, many species have turned out to be complexes of sibling lineages (Boisselier-Dubayle & Gofas, 1999; Carreras-Carbonell, Macpherson & Pascual, 2005; Calvo *et al.*, 2009; Sá-Pinto *et al.*, 2010; Xavier *et al.*, 2011). Molluscan taxonomy, in particular, historically has been almost entirely based on shell characters, which are still commonly used even though morphological variation caused by adaptation to environmental pressures has been documented (Vermeij, 1978). Consequently, traditional shell-based taxonomic practice has proved to be problematic for molluscs, and particularly for morphologically diverse groups. Therefore, in several of the most recent studies on marine molluscs, the combination of molecular and traditional approaches has proven to be successful to resolve taxonomically doubtful groups (e.g. Puillandre *et al.*, 2009; Claremont *et al.*, 2011; Zou, Li & Kong, 2012).

The genus *Ocenebrina* Jousseaume, 1880 is widely distributed in the Mediterranean Sea, northeastern Atlantic and northeastern Pacific, with species commonly called small mussel drills. It includes 23 currently accepted living species: 16 in the northeastern Atlantic and Mediterranean and seven in the northeastern Pacific (WoRMS: Appeltans *et al.*, 2012, at <http://www.marinespecies.org> – accessed on 13 March 2013). The Pacific species are often attributed to other genera, such as *Ocenebra* Gray, 1847 and *Urosalpinx* Stimpson, 1865 (two of the c. 29 genera in the subfamily Ocenebrinae Cossmann, 1903: see, for example, Radwin & D'Attilio, 1976; Vermeij & Vokes, 1997; but see WoRMS – accessed on 13 March 2013 – for an updated listing), and their relationships will be dealt with in an ongoing study on the phylogenetics of the Ocenebrinae Cossmann, 1903. The northeastern Atlantic and Mediterranean species are traditionally divided into two groups of morphological affinities: the *O. aciculata* complex and the *O. edwardsii* complex. The *O. aciculata* complex, recently revised

by Crocetta *et al.* (2012), includes *O. aciculata* (Lamarck, 1822), *O. corallinoides* Pallary, 1912 and *O. reinai* Bonomolo and Crocetta, 2012. The *O. edwardsii* complex includes the following species: *O. edwardsii* (Payraudeau, 1826), *O. carmelae* Cecalupo, Buzzurro & Mariani, 2008, *O. helleri* (Brusina, 1865), *O. hispidula* Pallary, 1904, *O. hybrida* (Aradas & Benoît, 1876), *O. ingloria* (Crosse, 1865), *O. inordinata* (Houart & Abreu, 1994), *O. leukos* Houart, 2000, *O. miscowichae* Pallary, 1920, *O. nicolai* Monterosato 1884, *O. paddeui* Bonomolo & Buzzurro, 2006, *O. piantonii* Cecalupo, Buzzurro & Mariani, 2008 and *O. purpuroidea* Pallary, 1920 (see Table 2 for a state of the art nomenclature). *Ocenebrina edwardsii* is traditionally considered to be a common species and one of the most variable European muricids, as witnessed also by the existence of numerous names established for the various morphotypes (see Table 2), whose status has been debated extensively (see, for example, Houart, 2001). Its distribution spans the entire Mediterranean Sea and, in the Atlantic Ocean, it is recorded up to the Bay of Biscay and to the Canary Islands. The shell reaches (and sometimes exceeds) 20 mm in length with up to 5–5.5 teleoconch whorls, has a sealed siphonal canal, five to six small denticles on the internal part of the outer lip and variable coloration. The protoconch is paucispiral (1.25–1.75 whorls) and its morphology is similar to that of *O. aciculata*. Therefore, a development similar to that described for *O. aciculata* by Franc (1940) can be assumed, i.e. entirely intracapsular or with a very short pelagic phase.

The combination of the high morphological variability of the shell among and within species over a wide geographical range (see Supporting Information Figs S4–S6) has contributed to the confused taxonomy in the *O. edwardsii* complex. Here, we have applied DNA-based methods to a sample including 243 specimens from the northeastern Atlantic and the Mediterranean Sea (including the area of the type locality of *Purpura edwardsii*, Corsica) in order to define the limits of this species and check for the presence of cryptic lineages. We also included specimens morphologically ascribed to some of the nominal species of the same complex to investigate their relationships with *O. edwardsii*: *O. ingloria* from the Venice Lagoon, *O. hispidula* from the Gulf of Gabès, *O. carmelae* and *O. piantonii* from the Kerkennah Islands, *O. leukos* from the Canary Islands and *O. miscowichae* from southern Morocco. In addition, we also processed specimens assigned in the recent literature to *O. cf. nicolai* and *O. nicolai* from Spain and Portugal (Rolán, 1983; Afonso *et al.*, 2011; Gofas, 2011), although their morphology was not perfectly matching that of the nominal taxon. Using three

markers [mitochondrial cytochrome oxidase subunit I (COI) and 16S, and the nuclear internal transcribed spacer 2 (ITS2)], we applied several methods of species delimitation and used the results to discuss the status of the *O. edwardsii* complex. We used the ocinebrine *Nucella lapillus* (Linné, 1758) as an outgroup in the phylogenetic analyses, and also included specimens of *Ocinebrina aciculata* and *O. reinai* as recently defined by Crocetta *et al.* (2012).

MATERIAL AND METHODS

SAMPLE DATA

The specimens used in this work were collected by hand, snorkelling or scuba diving. Sample localities are summarized in Table 1 (see also Figs 1 and 4) with their tissue-processing codes. Each specimen was identified on collection and fixed in 96–100% ethanol. A piece of tissue was later dissected from the foot for DNA extraction.

DNA SEQUENCING AND ALIGNMENT

DNA extraction was performed after tissue digestion in proteinase K using a phenol–chloroform protocol (Hillis, Moritz & Mable, 1996) with slight modifications as described by Oliverio & Mariottini (2001b). A fragment of the mitochondrial COI was amplified using the universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994), part of the mitochondrial 16S was obtained with the primers 16SA (Palumbi *et al.*, 2002) and CGLeu^{UUR} (Hayashi, 2005), and the entire nuclear ITS2 rRNA gene was amplified using the primers ITS-3d and ITS-4r (Oliverio & Mariottini, 2001a). Polymerase chain reaction (PCR) amplifications were performed in 25 µL containing 2.5 µL of BIOLINE 10× buffer, 0.5 µL of 10 mM deoxynucleoside triphosphates (dNTPs) mix, 0.4 µL of each primer (10 mM), 2.5–3 µL of 50 mM MgCl₂, 1 U of BIOLINE TaqPolymerase and 0.5–1 µL of genomic DNA.

The DNA fragments were amplified with an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C (COI and 16S) and 62 °C (ITS2) for 40 s and extension at 72 °C for 50 s. These cycles were followed by an extension at 72 °C for 10 min. PCR products were purified with the ExoSAP protocol. A mix of 2 µL containing 20 U µL⁻¹ Exonuclease I (New England Biolabs, Ipswich, MA, USA) and 1 U µL⁻¹ Shrimp Alkaline Phosphatase (Roche, Basel, Switzerland) was used to purify 5 µL of PCR product. Fragments were sequenced by Macrogen Inc. (Seoul, South Korea) using the same PCR primers in BigDye terminator cycling conditions (Applied Biosystems, Carlsbad, CA, USA). Reacted products were purified

using ethanol precipitation and run using an automatic sequencer AB3730XL (Applied Biosystems).

Forward and reverse sequences were assembled and reciprocally edited with Sequencher (v. 4.1.4; Gene Codes Corporation, Ann Arbor, MI, USA). The COI dataset was aligned manually and translated into amino acids to check for stop codons. The 16S and ITS2 dataset was aligned with MAFFT (Katoch *et al.*, 2002) using the Q-INS-i algorithm (Katoch & Toh, 2008), which accounts for secondary structures in the sequences. The nucleotide substitution models were selected by jModelTest (Posada, 2008) using the Bayesian Information Criterion (Schwarz, 1978). The likelihood scores for 88 possible substitution models were calculated with PhyML (Guindon & Gascuel, 2003) using a tree obtained separately for each model with BIONJ (Gascuel, 1997) employing a JC model (Jukes & Cantor, 1969).

SPECIES DELIMITATION

Three methods of species delimitation were applied to identify Molecular Operational Taxonomic Units (MOTUs) (Blaxter, 2004) in the COI dataset: the Automatic Barcode Gap Discovery (ABGD) method (Puillandre *et al.*, 2012a), the Generalized Mixed Yule-Coalescent (GMYC) technique (Pons *et al.*, 2006) and statistical parsimony network analysis (Posada & Crandall, 2001; Templeton, 2001).

ABGD is a distance-based method designed to detect the so-called 'barcode gap' in the distribution of pairwise distances calculated in a COI alignment (Puillandre *et al.*, 2012a, b). A distance value corresponding to the most reliable gap was used to group the sequences in MOTUs. The web-based ABGD program (available at <http://www.wabi.snv.jussieu.fr/public/abgd/>) was employed to generate a preliminary partition of sequences, using a distance matrix calculated from the COI dataset. The COI alignment was submitted and processed in ABGD (excluding the outgroup *O. miscowichae*) using the Kimura two-parameter (K2P) model (Kimura, 1980) and the following settings: a prior for the maximum value of intraspecific divergence between 0.001 and 0.1, 25 recursive steps within the primary partitions defined by the first estimated gap, and a gap width of 0.1.

The GMYC method identifies the species–population boundary in a sample of multiple species and populations using a time-calibrated (ultrametric) tree and a likelihood statistic (Pons *et al.*, 2006). The boundary represents the shift of the tree branching rates from a Yule (interspecific) to a coalescent (intraspecific) model, and is estimated as the likelihood peak of the transition along the branches. An ultrametric tree based on the COI alignment (excluding the outgroup *O. miscowichae*) was generated with

Table 1. Collection sites with relevant BAU voucher codes, number of assayed specimens (N), their preliminary identification on collection and the Molecular Operational Taxonomic Unit (MOTU) in which they were included, according to the four-MOTUs (I–IV) and eight-MOTUs (A–H) hypotheses (see text). Vouchers from each sampling site are figured in Supporting Information Figs S4–S6.

BAU voucher code	N	Sampling locality, environmental data	Coordinates	A priori morphological identification	MOTU
902	5	Italy, Venice Lagoon, low tide on/under stones	45°17'18"N, 012°16'18"E	<i>O. ingloria</i>	IV H
903	5	Tunisia, Djerba Island, south-east of Ajdjm, 0.5 m depth, amidst <i>Posidonia</i> rhizomes	33°41'49"N, 010°47'25"E	<i>O. hispidula</i>	III D
904	5	Italy, Augusta, Megara harbour, 0–1 m depth, on/under stones	37°13'50"N, 015°13'27"E	<i>O. educardsii</i>	II C
905, 923	3, 4	Italy, Marzamemi, harbour, 0–1 m depth, on/under stones	36°44'30"N, 015°07'03"E	<i>O. educardsii</i>	II C
907	11	Italy, Stracusa, Punta Asparano, 1 m depth, cave, amidst calcareous algae	36°59'32"N, 015°15'41"E	<i>O. educardsii</i>	II C
909	16	Italy, Lampetusa Island, Cala Francese, low depth, in cave	35°29'43"N, 012°37'26"E	<i>O. educardsii</i>	III E, G
920, 1299	3, 7	Italy, Pozzoli, 'o vajjone' harbour, 0.5 m depth, on/under rocks amidst algae	40°49'18"N, 014°07'08"E	<i>O. educardsii</i>	III E, G
921	2	Italy, Bacoli, Punta Penitola, 0.5 m depth, amidst mussels	40°47'35"N, 014°05'11"E	<i>O. educardsii</i>	III E
922	7	Italy, Marina di Schiavonea, 0–1 m depth, on/under rocks and amidst mussels	39°38'29"N, 016°34'00"E	<i>O. educardsii</i>	IV H
924	4	Italy, Marsala, 0–1 m depth, under stones	37°47'50"N, 012°25'45"E	<i>O. educardsii</i>	III E
927	6	Greece, Limnos Island, 0–1 m depth, on/under stones	39°49'58"N, 025°15'55"E	<i>O. educardsii</i>	I B
936	3	Greece, Heraklion, Lygaria, Crete, 0–1 m depth, on/under stones	35°23'56"N, 025°01'41"E	<i>O. educardsii</i>	I B
965, 1058, 1059, 1061, 1062	1, 1, 10, 3, 2	Spain, Bayona, Cabo Silleiro, low tide, on rocks	42°06'40"N, 008°53'57"W	<i>O. educardsii</i> (partim)	III D
959	4	Croatia, Crvar, 0–1 m depth, on/under stones	45°27'14"N, 013°31'03"E	<i>O. nicolai</i> (partim) sensu Rolán (1983)	IV H
960	6	Croatia, Katoro, 0–1 m depth, on/under stones	45°27'56"N, 013°30'31"E	<i>O. educardsii</i>	IV H
961	4	Italy, Gallipoli, Punta Pizzo, 0–1 m depth, on/under stones	40°00'01"N, 017°59'37"E	<i>O. educardsii</i>	IV H
962	3	Italy, Muggia, Punta Solite, 0–1 m depth, on/under stones	45°36'20"N, 013°43'10"E	<i>O. educardsii</i>	IV H
963, 1033	1, 5	Spain, Vigo, low tide, on rocks	42°13'52"N, 008°42'44"W	<i>O. educardsii</i> (partim)	III D
969	2	Italy, Marettimo Island, Cammello shoal, 10 m depth, under stones	37°59'21"N, 012°13'55"E	<i>O. educardsii</i>	III E
1026	4	Italy, Camponarino, 0–1 m depth, on/under stones	40°18'03"N, 017°30'13"E	<i>O. educardsii</i>	III C
1029	2	Croatia, Umag, Sol Stella Maris, 0–1 m depth, on/under stones	45°27'02"N, 013°30'45"E	<i>O. educardsii</i>	IV H
1044	5	Tunisia, Sfax, fishing nets residuals, from <i>Posidonia</i> meadows	34°42'N, 010°42'E	<i>O. hispidula</i>	III D
1045	5	Italy, Pugnochiuso, 0–1 m depth, on/under stones	41°47'14"N, 016°11'22"E	<i>O. educardsii</i>	IV H
1046, 1293	5, 7	Italy, Pellestrina, Venice Lagoon, low tide under stones	45°15'13"N, 012°17'57"E	<i>O. educardsii</i>	III F
1048	5	Tunisia, Djerba Island, Boji Kastil, 0.5 m depth, amidst <i>Posidonia</i> rhizomes	33°41'00"N, 010°51'27"E	<i>O. hispidula</i>	III D
1049	6	Italy, Stracusa, harbour, 0.5 m depth, amidst mussels	33°04"N, 015°17'E	<i>O. educardsii</i>	II C
1050	5	Croatia, Murter Island, 1–10 m depth, on/under rocks	43°47'48"N, 015°36'34"E	<i>O. educardsii</i>	IV H
1051	1	Tunisia, Kerkennah Islands, Ra's Bu Numah, 0.5–0.8 m depth, amidst <i>Posidonia</i> rhizomes	34°47'00"N, 011°12'55"E	<i>O. pianonii</i>	I A
1052	1	Tunisia, Kerkennah Islands, Borj el-Hissar, –0.5 m, under stones	34°42'42"N, 011°09'05"E	<i>O. curmiae</i>	I A
1053	1	Tunisia, Kerkennah Islands, Ra's Amrit, –0.5 m, near a small stone	34°40'60"N, 011°07'00"E	<i>O. hispidula</i>	III D
1054	1	Tunisia, Kerkennah Islands, near Kraien, low tide in very quiet waters, sandy beach	34°43'N, 011°11'E	<i>O. hispidula</i>	III D
1055	7	Greece, Milos Island, low depth, on/under rocks	36°39'43"N, 024°25'41"E	<i>O. educardsii</i> (partim)	III D
1063	4	Spain, O Grove, low depth, on/under rocks	42°30'N, 008°52'W	<i>O. nicolai</i> (partim) sensu Rolán (1983)	III D
1064	1	Croatia, Cres Island, Punta Kriza, low depth, on/under rocks	44°38'29"N, 014°30'20"E	<i>O. educardsii</i>	IV H
1065	5	Portugal, Peniche, Peniche de Cima beach, very low tide, rocky bottom, under and on rocks	39°22'15"N, 009°22'18"W	<i>O. educardsii</i>	III D
1106	4	Italy, Sardinia, Santa Maria Navarrese, 3 m depth, on rocks	39°59'15"N, 009°41'26"E	<i>O. educardsii</i>	III E
1107	3	Italy, Zannone Island, 10 m depth, on rocks	40°57'54"N, 013°03'26"E	<i>O. educardsii</i>	III E
1109	3	Italy, Marettimo Island, Punta Mugnone, 18 m depth, under stones	37°59'15"N, 012°01'28"E	<i>O. educardsii</i>	III E
1280	5	Spain, Benalmádena, 1–5 m depth, on rocks	36°35'03"N, 004°31'07"W	<i>O. educardsii</i>	III D
1281	1	Spain, Calahonda, low tide, amidst rocks and algae	36°29'04"N, 004°41'08"W	<i>O. educardsii</i>	III D
1282	2	Spain, Barbate, low tide, amidst rocks	36°11'00"N, 005°56'09"W	<i>O. nicolai</i> sensu Gofas (2011)	III D
1290	2	Spain, Tenerife Islands, La Caleta, low depth, on/under rocks,	22°28'00"N, 016°47'00"W	<i>O. taitoku</i>	III D
1291	3	Spain, Tenerife Islands, Punta de Teno, –15 m	28°20'31"N, 016°55'23"W	<i>O. educardsii</i>	III D
1294	1	Portugal, off Ponta da Piedade, low depth, on/under rocks	37°03'32"N, 008°38'54"W	<i>O. nicolai</i> sensu Afonso et al. (2011)	III D
1295	6	Italy, Cephalo, low tide on rocks	38°00'57"N, 014°06'55"E	<i>O. educardsii</i>	III E
1296	3	France, Corsica, Porto Vecchio, Saint-Cyprien beach, 0–0.5 m depth, on granitic rocks	41°36'N, 009°17'E	<i>O. educardsii</i>	III E
1298	6	Greece, Kavala, –0.5 m, under rocks	40°54'49"N, 024°22'42"E	<i>O. educardsii</i>	II B
1300	4	Italy, Palinuro, tide level amidst algae	40°02'07"N, 015°17'07"E	<i>O. educardsii</i>	III G
1308	3	Italy, Sardinia, Cagliari, Marina Piccola, harbour, –2 m, on rocks	39°11'28"N, 009°09'53"E	<i>O. educardsii</i>	III E
1133	5	Morocco, Dakhla, low water, beach, on rocks	23°41'N, 015°55'W	<i>O. miswickhae</i>	–
1060	6	Spain, Bayona, Cabo Silleiro, low tide, on rocks	42°06'40"N, 008°53'57"W	<i>O. aciculata</i>	–
1037, 1042	1, 1	Italy, Tor Paterno MPA, –25 m	41°36'18"N, 012°20'30"E	<i>O. aciculata</i>	–
1038	1	Italy, Tor Paterno MPA, –25 m	41°36'21"N, 012°20'28"E	<i>O. reitani</i>	–
1039	1	Italy, Tor Paterno MPA, –20 m	41°36'13"N, 012°20'30"E	<i>O. reitani</i>	–
187	1	UK, Portobello, intertidal	55°57'N, 030°6'W	<i>Nucella lapillus</i>	–

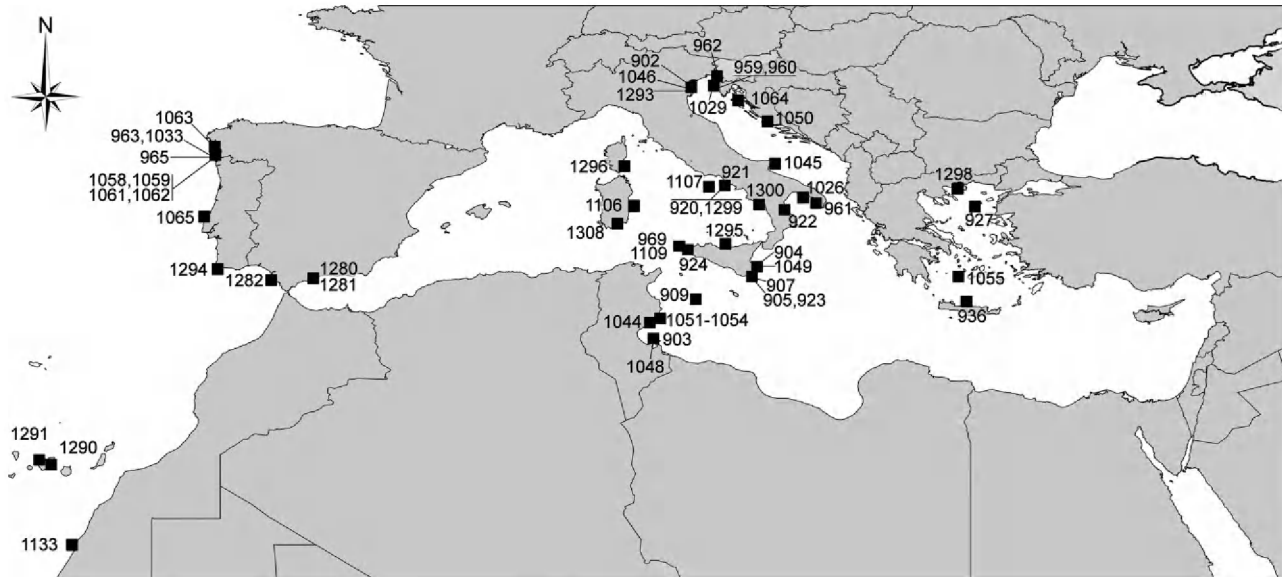


Figure 1. Location map of the sampling sites. Numbers of the sites as in Table 1.

BEAST (Drummond & Rambaut, 2007), using the substitution model proposed by jModelTest. A relaxed lognormal clock, a substitution rate fixed to one (no calibrations were used as our aim was to estimate the branching rates only) and a constant coalescent prior (which is thought to be more conservative than a Yule prior for species delimitation: see Monaghan *et al.*, 2009) were used. Four BEAST runs were performed, each with 10^8 generations sampling every 10^4 . Two samples of 10^4 trees were obtained, and convergence was evaluated, reading the log files with Tracer (Rambaut & Drummond, 2003), to verify that the effective sample size (ESS) values were > 200 . Trees from all the runs were combined with LogCombiner and then summarized in a maximum credibility tree with TreeAnnotator (both included in the BEAST package). The consensus tree was analysed, applying the single-threshold GMYC function from the SPLITS package (Ezard, Fujisawa & Barraclough, 2009) in R (<http://www.R-project.org>). Having found no incongruence between ITS2 and COI single-gene analyses (see below), we also analysed with the same settings the combined COI + ITS2 dataset, where the clock and substitution model were unlinked for each gene, and the substitution models suggested in jModelTest were used.

The statistical parsimony network analysis calculates the maximum number of mutational steps constituting a parsimonious connection between two haplotypes (Posada & Crandall, 2001; Templeton, 2001). The haplotypes are then joined into networks following the algorithms proposed by Templeton, Crandall & Sing (1992), and those separated by more

mutational steps (i.e. with probability of secondary mutations exceeding 5%) remain disconnected. This method has been used previously to differentiate species in a mixed sample (Pons *et al.*, 2006; Hart & Sunday, 2007). It was applied to our COI dataset (excluding the outgroup *O. miscowichae*) using TCS (Clement, Posada & Crandall, 2000) with a 95% limit of tolerance.

We also analysed the ITS sequences for fixed sites between MOTUs defined in the species delimitation tests using DNASp (Librado & Rozas, 2009)

PHYLOGENETIC ANALYSIS

Bayesian phylogenetic trees for each single gene and for the combined COI + ITS2 and COI + ITS2 + 16S datasets were estimated using MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Sequences of *Nucella lapillus* (Linnaeus, 1758) were used to root the trees and, where available, sequences of *O. aciculata*, *O. reinai* and *O. miscowichae* were included to test their relationships with the species included in this study. Two Metropolis-coupled Markov chain Monte Carlo (MC³) algorithms, with four chains each, were run for 8×10^7 generations using the substitution models selected by jModelTest, and sampling every 8×10^3 . Convergences of the chains were evaluated by plotting values of the standard deviation of average split frequencies, and with the Potential Scale of Reduction Factor (PSRF) (Gelman & Rubin, 1992). All the analyses in MrBayes were computationally intensive, and thus run on the University of Oslo Biportal (Kumar *et al.*, 2009).

To test for incongruence among trees obtained with single-gene analyses, we compared clades recovered with high support in each topology. Conflict among clades with high support can be seen as evidence for divergent evolutionary histories, and can be used as an argument against multiple-gene phylogenetic analyses. However, we found no conflict among single-gene trees, and combined the datasets to estimate Bayesian topologies using COI + ITS2 and COI + ITS2 + 16S.

RESULTS

SEQUENCE ANALYSIS

We obtained 208 original sequences of COI, 58 of 16S and 155 of ITS2, and included in our analyses 38 COI sequences of *O. cf. edwardsii* and *O. hispidula* from a previous study (Barco, Corso & Oliverio, 2013). Ten COI sequences of *O. aciculata* and *O. reinai* from Crocetta *et al.* (2012) were included in the phylogenetic analyses, together with six new COI sequences of *O. aciluata* from Spain (BAU 1060). The COI alignment spanned 658 bp (no stop codon detected), 167 of which were variable and 133 were parsimony informative; the 16S alignment comprised 763 bp, 83 of which were variable and 58 were parsimony informative; the ITS2 alignment comprised 559 sites, 157 of which were variable and 66 were parsimony informative. Accession numbers for the sequences are provided in Supporting Information Table S1. The substitution models selected by jModelTest were the TPM2uf + I + Γ (Kimura, 1981) for COI, HKY + Γ (Hasegawa, Kishino & Yano, 1985) for ITS2 and TIM3 + I + Γ (Posada, 2003) for 16S. The distances for the ABGD analysis on the COI dataset were obtained using the K2P model (Kimura, 1980) implemented in the online software.

SPECIES DELIMITATION

The ABGD method on the 236 COI sequences proposed nine distinct groupings ranging from 67 MOTUs to a single one depending on the value of the maximum intraspecific divergence used as a boundary between inter- and intraspecific distance values. The results obtained for K2P values lower than 0.012 were discarded for the unrealistic assumption of more than 10 species. The histogram of the pairwise distances (Fig. 2) shows a multimodal distribution for the frequencies, with a relevant minimum for values of K2P distance between 0.020 and 0.025, corresponding approximately to the partitions 15–17 (K2P distance = 0.014–0.021, eight MOTUs) and 18 (K2P distance = 0.026, four MOTUs).

All of the four BEAST runs reached ESS values of > 200, and the 'burnin' was set for one-half of the

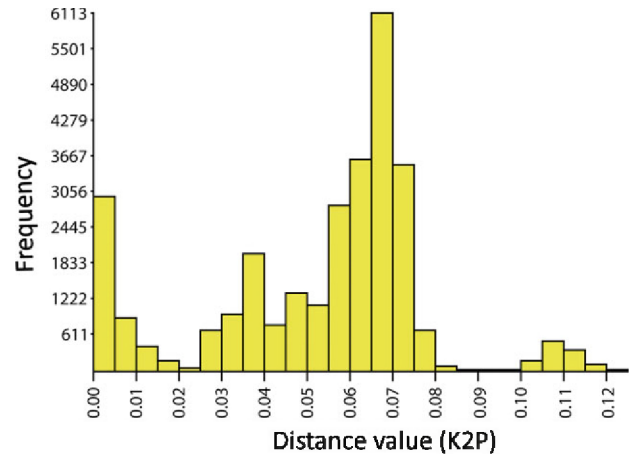


Figure 2. Histogram of the frequency of Kimura two-parameter (K2P) genetic divergence in all pairwise comparisons of cytochrome oxidase subunit I (COI) sequences.

generations. A final sample of 10^4 trees was used to obtain the maximum clade credibility tree shown in Figure 3. The single-threshold model in GMYC was significantly better than the null model of no shift in branching rates ($P < 0.001$). The GMYC method proposed eight MOTUs (confidence interval, 2–18). In addition, in the combined dataset (COI + ITS2), the GMYC method performed better than the null model, but the dataset was divided into only three MOTUs (confidence interval, 1–36; Supporting Information Fig. S1).

The 238 COI sequences comprised 70 distinct haplotypes, 37 of which were unique. Using a 95% cut-off, the haplotypes were grouped by statistical parsimony analysis into nine independent networks, considered here as equivalent to nine MOTUs (Fig. 3).

The MOTUs defined by the three species delimitation methods in the eight-MOTUs scenario, based on the COI alignment, are named I–IV (four-MOTUs scenario: ABGD) and A–H (eight-MOTUs scenario: ABGD, GMYC, TCS) in Figure 3. Concordance was found among the three analyses, with only a single difference: group D was recovered as a single MOTU in the ABGD and GMYC analyses, but split into two by TCS (D1 and D2). In the two-genes (COI + ITS2) GMYC test, the threshold time for the coalescent/Yule boundary was lower (–0.044) than in the single-gene test (–0.01), resulting in the groups A + B + C and D + E + F + G, respectively, clustered into two MOTUs (Fig. S1).

Conserved sites in ITS2 were calculated for the MOTUs suggested in the species delimitation analysis (data not shown). Fixed differences were found only between more distantly related groups (e.g. B–D1, B–D2 and B–F), whereas most sister groups

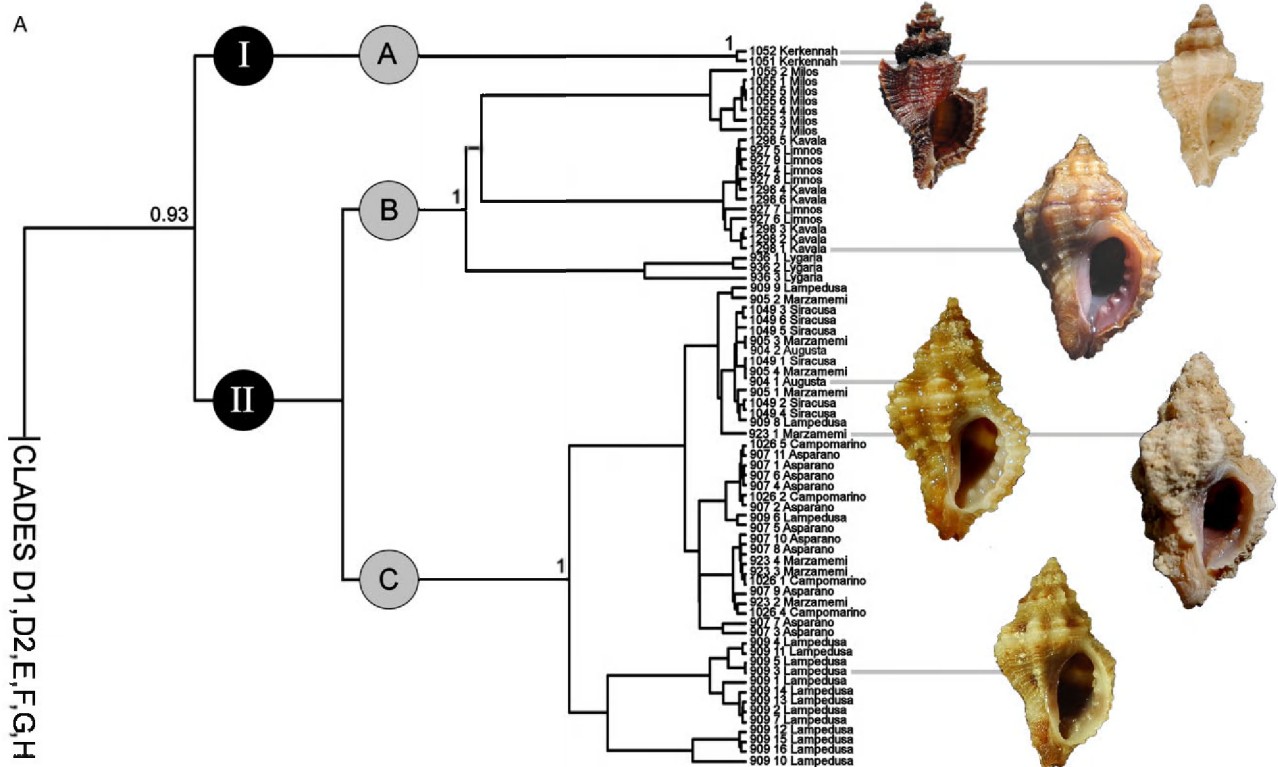


Figure 3. Consensus tree of a final sample of 10^4 trees obtained by BEAST on the cytochrome oxidase subunit I (COI) alignment. The black bars on the right delimit the Molecular Operational Taxonomic Units (MOTUs) as defined by the three species delimitation methods employed [Automatic Barcode Gap Discovery (ABGD), Generalized Mixed Yule-Coalescent (GMYC) and TCS]. Posterior supports > 0.95 are reported, and only for the nodes subtending the MOTUs. Voucher shells for each MOTU are figured (shells not to scale). 3A, MOTUs A–C; 3B, MOTUs D1–H.

showed no fixed differences, usable as diagnostic for each MOTU.

PHYLOGENETIC ANALYSIS

In the MrBayes analysis, convergence was reached by the sampling chains in almost all analyses, as shown by the average standard deviation of split frequencies lower than 0.01 and by PSRF values of 1.00 for each parameter estimated by the MC³ algorithm. However, for the single-gene ITS2 analysis, the convergence was difficult to reach even after setting a starting tree, increasing the number of generations, the number of MCMC chains or the λ value. In all the analyses, we employed a 'burnin' of about 25% of the sampled trees, whereas, in the ITS2 analysis, we used a 50% 'burnin'.

The single-gene Bayesian analyses produced three topologies (Supporting Information Fig. S2). In the COI and ITS2 analyses, using *Nucela lapillus* as an outgroup, the *O. aciculate* complex (*O. aciculata* and *O. reinai*) and the *O. edwardsii* complex were reciprocally monophyletic. In particular, in the COI analysis,

O. miscowichae was defined as the sister taxon to the remaining lineages of the *O. edwardsii* complex. We obtained a high congruence between the COI and 16S trees (Fig. S2A,C), whereas the topology of ITS2 (Fig. S2B) was divergent and less resolved. Groups A–H of the BEAST-GMYC test were also obtained in the COI and 16S trees with high posterior probability (> 0.96), except for group G, which was unresolved in the 16S tree. In the ITS2 tree, none of the clades obtained with the other alignments was recovered. The limited amount of sequence variability observed in ITS2 probably prevented clear resolution of the phylogenetic signal in this alignment. We found no incongruence among highly supported clades in the single-gene trees, and thus also computed the combined-gene trees. In the combined-gene analyses (COI + ITS2, Supporting Information Fig. S3A; COI + ITS2 + 16S, Fig. S3B), we obtained two topologies similar to those of the COI and 16S single-gene analyses. In both cases, groups E and G of the BEAST-GMYC test had a moderate support (between 0.90 and 0.95), whereas all the others had high support values (> 0.96).

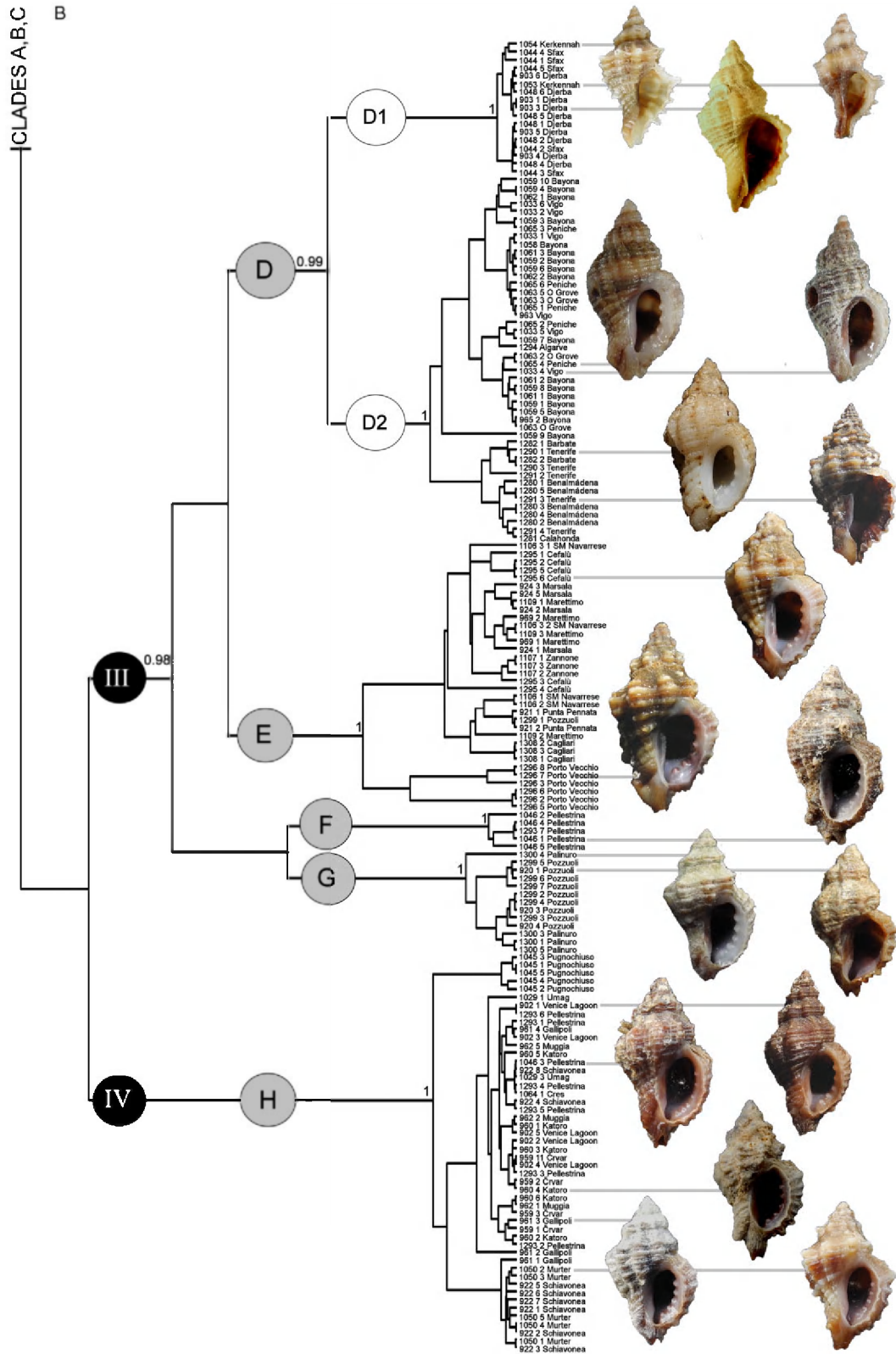


Figure 3. Continued.

DISCUSSION

As a starting point to define the threshold between intraspecific and interspecific pairwise comparisons, we searched those values (or range of values) that separated sympatric samples falling in distinct clades. The minima in the multimodal distribution for the frequencies of the pairwise distances (Fig. 2) corresponding to values higher than 0.031, defined in ABGD, partition with a single species for all of our samples. This is an unrealistic scenario which would classify the Tunisian specimens identified as *O. piantoni* and *O. carmelae* as conspecific with the sympatric specimens of *O. hispidula*, despite a K2P distance of >0.09 in sympatry. A single-species hypothesis would also produce a complete lack of geographical pattern in the phylogenetic trees, with northern Sicily and Tyrrhenian samples more closely related to Adriatic and (some) Tunisian ones than to southern Sicily and Aegean samples. Conversely, the values of the K2P distance between 0.020 and 0.026 corresponded approximately to the partitions 15–17 (K2P distance = 0.014–0.021, eight MOTUs) and 18 (K2P distance = 0.026, four MOTUs). The eight-MOTUs scenario was the same as that depicted by the GMYC method, and the four-MOTUs scenario was within the confidence limits (2–18 MOTUs) of the GMYC results.

For COI, the K2P distance range representing the intra-/interspecific boundary in the eight-MOTUs/ four-MOTUs scenarios (0.014–0.026) is similar to that obtained from the analysis of the COI sequences in other studies on marine gastropods (Castelin *et al.*, 2010; Reid, Dyal & Williams, 2010; Williams *et al.*, 2011; Nuñez *et al.*, 2012; Puillandre *et al.*, 2012b). This suggests that our COI distance values represent a reliable boundary between congeneric *Ocinebrina* species. However, deep mitochondrial divergences within other mollusc species have been repeatedly observed (e.g. Quattro *et al.*, 2001; Wilson, Schrödl & Halaných, 2009), raising the need for support with nuclear data before taking taxonomic decisions. Several previous studies have provided evidence of a general concordance between results from COI and ITS2 sequence analyses and their usefulness in the species delimitation of molluscs (Calvo *et al.*, 2009; Puillandre *et al.*, 2010; 2011). However, it is also known that too large or too small an intraspecific variation of ITS2 may confound the detection of cryptic species (Vilas, Criscione & Blouin, 2005).

According to the species delimitation methods based on the COI dataset, a set of lineages that possibly represent cryptic species emerged within the *O. edwardsii* complex. A final taxonomic revision of the complex, also including the description of new species, will depend on the detection of a genetic

distinction also at the nuclear level, and, eventually, on the identification of diagnostic morphological characters. Unfortunately, the lack of objectively distinguishable shell characters (at least at this point of our study), as well as the absence of a defined phylogenetic pattern in the selected nuclear sequences, prevented us from providing such a taxonomic revision. Therefore, we discuss the pattern emerging from the mitochondrial data, and its congruence with the preliminary shell-based identifications, under the assumption that, in our case, the mitochondrial phylogenetic signal is more reliable than the ITS2 one.

The small mussel drills of the *O. edwardsii* complex are common northeastern Atlantic and Mediterranean gastropods displaying striking morphological variability and, consequently, a complicated taxonomic history. As a probable consequence of their variability, numerous nominal taxa have been erected based exclusively on shell morphology for these gastropods (Houart, 2001; Cecalupo *et al.*, 2008). Thirteen species based on a purely morphological approach are accepted currently in the northeastern Atlantic–Mediterranean area (Table 2). The specimens used in our analysis were classified morphologically on collection into seven of these nominal species (*O. carmelae*, *O. edwardsii*, *O. hispidula*, *O. ingloria*, *O. leukos*, *O. miscowichae*, *O. piantonii*). Of these, *O. miscowichae* proved to be the sister species to the *O. edwardsii* complex (when *Nucella lapillus* was used as an outgroup, and *O. aciculata* was sister to the *O. miscowichae*–*O. edwardsii* pair). In our dataset, specimens morphologically ascribed to *O. helleri*, *O. nicolai* and *O. paddeui* (Table 2) were not available for DNA extraction. Their inclusion in a future dataset will probably affect the taxonomic and nomenclatural patterns. Within the *O. edwardsii* complex, our analyses proposed a number of MOTUs ranging from four to eight, and a preliminary hypothesis of species delimitation only partially concordant with our *a priori* identifications.

THE *OCINEBRINA EDWARDSII* COMPLEX

In the four-MOTUs scenario, supported by the ABGD method only (yet within the confidence limit of the GMYC analysis), the four major lineages (I–IV) comprised the following samples:

MOTU-I: specimens morphologically identified as *O. piantonii* and *O. carmelae* from the Gulf of Gabès.

MOTU-II: specimens morphologically identified as *O. edwardsii* from the Aegean Sea, Ionian Sea, southwestern Sicily and Lampedusa Island.

MOTU-III: specimens morphologically identified as *O. edwardsii* from the Tyrrhenian Sea (including

Table 2. List of available nominal taxa from the northeastern Atlantic and the Mediterranean currently included in the genus *Ocenebrina*. Names currently considered as valid are given in bold. The type locality or, in substitution (in square brackets), the list of localities reported in the original description and the location of the type material are given when known. We have excluded unavailable names, such as infraspecific names (ICZN, 1999: Art. 45.5, 45.6) or *nomina nuda* (ICZN, 1999: Art. 12 and Glossary), but also all names introduced by Settepassi (1977), which has sometimes been considered as a consistently non-binomial work, under ICZN, 1999: Art. 11.4 [see, for example, WoRMS (2012) for *Ocenebrina eduardisi* var. *fasciatus* Settepassi, 1977. Accessed through: World Register of Marine Species at <http://www.marinespecies.org/aphia.php?p=taxdetails&id=403955> on 13 March 2013]

Nominal taxon	Type locality [listed localities]	Type material	Currently accepted as	Notes
<i>Murex aciculatus</i> Lamarck, 1822: 176	Vannes area (France)	Not known	<i>O. aciculata</i>	Type species of <i>Ocenebrina</i> Jousseaume, 1880 and <i>Corallinita</i> Bucquoy & Dautzenberg, 1882 (in Bucquoy, Dautzenberg & Dollfus, 1882: 24)
<i>Murex acrisius</i> Nardo, 1847: 59–60	Gulf of Venice (Italy)	14 syntypes MSNVE 21880 (Crocetta et al., 2012)	<i>O. aciculata</i>	Originally referring to: Chiereghin (unpublished*); figs. 713–714. Subjective junior synonym (Crocetta et al., 2012)
<i>Ocenebrina eduardisi</i> [sic] var. <i>apiculata</i> Pallary, 1902a: 314	Tanger (Morocco)	Not known	<i>O. eduardisi</i>	
<i>Murex baeticus</i> Reeve, 1845: pl. 32 (fig. 162)	Not known	3 syntypes NHMUK 1972024 – one in Houart (2001) as BMNH 1621845	<i>O. eduardisi</i>	Probable subjective senior synonym of <i>Murex hybridus</i>
<i>Ocenebrina buzzurroi</i> Cecalupo & Mariani, 2008: in Cecalupo et al., 2008: 90, pl. 43 (figs. 1–7)	Borj el Hissar (Tunisia)	Holotype MNHM 33489 (Cecalupo et al., 2008)	<i>O. corallinooides</i>	Subjective junior synonym (Crocetta et al., 2012)
<i>Ocenebrina carmelae</i> Cecalupo, Buzzurro & Mariani, 2008: 98, pl. 48 (figs. 1–8)	Borj el Hissar (Tunisia)	Holotype MNHM 33491 (Cecalupo et al., 2008)	<i>O. carmelae</i>	New subjective junior synonym of <i>O. piantonii</i> (? = <i>Murex hybridus</i>)
<i>Amyxina compacta</i> Nordischeck, 1968: 140, pl. 23 (fig. 80.45)	Portovenere (Italy)	Holotype nr/1, Senckenberg Mus. (Houart, 2001)	<i>O. eduardisi</i>	
<i>Ocenebrina corallinooides</i> Pallary, 1912: 221, plate (fig. 48)	Sfax, Gulf of Gabès (Tunisia)	Not known	<i>O. corallinooides</i>	
<i>Murex corallinus</i> Scacchi, 1836: 12 (fig. 15)	Baia (Italy)	Neotype MZN Z7010 (Crocetta et al., 2012)	<i>O. aciculata</i>	Subjective junior synonym (Houart, 2001; Crocetta et al., 2012)
<i>Murex costulatus</i> Nardo, 1847: 55–56 (sp. 8)	Cres Island area (Croatia)	Not known	<i>O. aciculata</i>	Originally referring to: Chiereghin (unpublished*); figs. 693–694. Hereby first considered subjective junior synonym of <i>Fusus helleri</i> . Primary junior homonym of <i>Murex costulatus</i> Schröter 1805 and <i>Murex costulatus</i> Risso, 1826
<i>Ocenebrina eduardisi</i> [sic] var. <i>crassata</i> Pallary, 1902b: 12, pl. 1 (figs. 10–11)	Tanger (Morocco)	Not known	<i>O. eduardisi</i>	Hereby considered as belonging to the <i>Ocenebrina eduardisi</i> complex
<i>Ocenebrina cyclops</i> Monterosato, 1884: 112	Palermo (Italy)	Syntypes in MCZR (Settepassi, 1977)	<i>O. eduardisi</i>	Type species of <i>Dentocenebra</i> Monterosato, 1917 (= <i>Ocenebrina</i> Jousseaume, 1880)
<i>Purpura eduardisi</i> Payraudeau, 1826: 155, pl. 7 (figs. 19–20)	Corsica (France)	Not known	<i>O. hispidula</i>	Subjective junior synonym (Cossignani & Ardovini, 2011), hereby assigned to Cecalupo et al. (2008) since <i>Muricopsis erroneus</i> Settepassi, 1977 is unavailable (ICZN, 1999: Art. 11.4)
<i>Ocenebrina erronea</i> Cecalupo, Buzzurro & Mariani, 2008: 92, pls. 42 (figs. 5–6), 44 (figs. 7–16) and 45 (figs. 1–10)	Sfax and Kerkennah Islands (Tunisia)	Not designated	<i>O. hispidula</i>	Subjective junior synonym (Bonomo & Buzzurro, 2006; Cecalupo et al., 2008; Crocetta et al., 2012)
<i>Ocenebrina aciculata exilis</i> Houart, 2001: 19 (figs. 8–9), 51 (fig. 62), 62, 143 (figs. 167–168), 176 (figs. 324–325)	NW of Bou Grara Sea (Tunisia)	Holotype MNHN 0362 (Crocetta et al., 2012)	<i>O. corallinooides</i>	= <i>Murex hellerianus</i> Brusina, 1866: 63 (unnecessary spelling emendation: Houart, 2001)
<i>Fusus helleri</i> Brusina, 1865: 8	[Zadar, Šibenik, Hvar, Dubrovnik, Budval] (Croatia and Montenegro)	Not known	<i>O. helleri</i>	
<i>Ocenebrina eduardisi</i> [sic] var. <i>hispidula</i> Pallary, 1904: 231, pl. 7 (fig. 18)	Gulf of Gabès (Tunisia)	3 syntypes MNHN 1001 (Houart, 2001; Giannuzzi-Savelli et al., 2003; present paper)	<i>O. hispidula</i>	Probable subjective junior synonym of <i>Murex baeticus</i>
<i>Murex hybridus</i> Aradas & Benoit, 1876: 272, pl. 5 (fig. 9)	Palermo (Italy)	Not known	<i>O. hybrida</i>	
<i>Murex inconspicuous</i> Sowerby G.B. II, 1841: 5 (67), plates (figs. 81)	Jersey Island (Bailiwick of Jersey)	Not known	<i>O. aciculata</i>	Subjective junior synonym (Houart, 2001; Crocetta et al., 2012)
<i>Murex inglorius</i> Crosse, 1865: 213, pl. 6 (fig. 4)	Not known	Holotype MNHN 0933 (Fair, 1976; Houart, 2001; Giannuzzi-Savelli et al., 2003; present paper)	<i>O. ingloria</i>	
<i>Ocenebra inordinata</i> Houart & Abreu, 1994: 123, 129 (figs. 11–13)	Madeira Island	Holotype MIMF 25429 (Houart & Abreu, 1994)	<i>O. inordinata</i>	

<i>Ocinebrina labiosa</i> Monterosato, 1884: 112	[Chioggia and Venice] (Italy)	Presumably not existing	Originally referring to: Chiereghin (unpublished*); figs. 695–696.
<i>Murex</i> [sic] <i>labiosus</i> Nardo, 1847: 55–56 (sp. 9)	Kvarner Gulf (Croatia)	Presumably not existing	New subjective junior synonym of <i>Murex inglorius</i> (unpublished*); Originally referring to: Chiereghin (unpublished*); figs. 695–696. Currently accepted as <i>Ocenebra ernaceus</i> , and here considered as a member of the <i>Murex edwardsii</i> complex. Primary junior homonym of <i>Murex labiosus</i> Gray, 1828 and <i>Murex labiosus</i> Wood, 1828
<i>Ocinebrina leukos</i> Houart, 2000: 464 (figs. 7–10)	La Isleta, Lanzarote (Spain)	Holotype MNHN 0966 (Houart, 2000; present paper)	Spelling emended by Houart (1997)
<i>Ocinebrina miscovicichi</i> Pallary, 1920: 40, plate (figs. 5–6)	Essaouira (Morocco)	32 syntypes MNHN 0176 and 0177 (Houart, 2001; present paper)	
<i>Ocinebrina nicolai</i> Monterosato, 1884: 112	[Corsica, Sardegna, Lipari] (Italy and France)	Syntypes in MCZR (Settepassi, 1977)	
<i>Murex oromenus</i> Nardo, 1847: 57–58 (sp. 11)	Northern Adriatic Sea	Not known	Originally referring to: Chiereghin (unpublished*); figs. 699–700. It is probably a senior synonym of <i>Murex inglorius</i> , but its status as <i>nomen oblitum</i> (ICZN, 1999: Art. 23.9.1.2) should be verified
<i>Ocinebrina paddeui</i> Bonomolo & Buzzurro, 2006: 1 (figs. 1–6)	Off Capo Caccia, Alghero (Italy)	Holotype MNHN 29909 (Bonomolo & Buzzurro, 2006)	
<i>Murex pergeri</i> Brugnone, 1873: 10, plate (fig. 17)	African shores	Not known	Subjective junior synonym (Houart, 2001). Primary junior homonym of <i>Murex pergeri</i> Beyrich, 1854
<i>Murex edwardsii</i> [sic] f. <i>perignus</i> De Gregorio, 1885: 253	Mediterranean	Not known	Originally referring to: Baeuoy <i>et al.</i> (1882): pl. 2 (fig. 3). Subjective junior synonym (Houart, 2001)
<i>Murex edwardsii</i> [sic] f. <i>perillus</i> De Gregorio, 1885: 253	Mondello (Italy)	Not known	
<i>Ocinebrina pizantoni</i> Cecalupo, Buzzurro & Mariani, 2008: 96, pl. 49 (figs. 1–10), pl. 50	Borj el Hissar (Tunisia)	Holotype MNHM 33490 (Cecalupo <i>et al.</i> , 2008)	Probable subjective junior synonym of <i>Murex baeticus</i>
<i>Murex pistacia</i> Reeve, 1845: pl. 34, fig. 174	Not known	3 syntypes NHMUK 1972021 (Crocezza <i>et al.</i> , 2012)	Subjective junior synonym (Houart, 2001; Crocezza <i>et al.</i> , 2012)
<i>Murex pumilus</i> Küster, 1869 (in Küster & Kobelt, 1839–1878: 118, pl. 35; figs. 8–10)	Not known	Not known	Primary junior homonym of <i>Murex pumilus</i> Broderip, 1833 and <i>Murex pumilus</i> A. Adams, 1853
<i>Ocinebrina purpuroides</i> Pallary, 1920: 39, plate (figs. 16–17, 24)	Rabat (Morocco)	8 syntypes MNHN 0931 (Houart, 2001)	
<i>Ocinebrina reinali</i> Bonomolo & Crocezza, 2012: (in Crocezza <i>et al.</i> , 2012: 180, figs. 1 K-1 L, 2A-2F, 2I, 2 L, 3)	Prosida Island (Italy)	Holotype MNHN 24566 (Crocezza <i>et al.</i> , 2012)	
<i>Murex semicaudatus</i> Küster, 1869: 111, pl. 34 (figs. 6–7)	Not known	Not known	
<i>Murex subaciculatus</i> Locard, 1886: 164	Toulon (France)	Not known	Originally referring to: <i>Murex aciculatus</i> in Hidalgo (1870): pl. 13 (figs. 7–8); <i>Murex aciculatus</i> var. <i>curta</i> Bucquoy <i>et al.</i> (1882): page 25. Subjective junior synonym (Houart, 2001; Crocezza <i>et al.</i> , 2012)
<i>Fusus titii</i> Stossich, 1865: 31	Northern Adriatic Sea	Not known	Subjective junior synonym (Houart, 2001; Crocezza <i>et al.</i> , 2012)
<i>Ocenebra</i> [sic] <i>wardiana</i> Baker, 1891: 134, pl. 11 (fig. 5)	'Australia' (see discussion in Vokes, 1994)	Holotype CAS 20698 (Vokes, 1994)	Subjective junior synonym (Houart, 2001; Crocezza <i>et al.</i> , 2012)
<i>Murex ueinikauffianus</i> Crosse, 1866: 274, pl. 8 (fig. 4)	Zadar (Croatia)	Not known	Subjective junior synonym (Houart, 2001)

*Chiereghin S. (unpublished). *Descrizione de' crostacei, de' testacei e de' pesci che abitano le lagune e gofo veneto*. [An unpublished manuscript referred to by Nardo, (1847) who then made available several otherwise unavailable names.]

Corsica, type locality of *O. edwardsii*); specimens morphologically identified as *O. hispidula* (from the Gulf of Gabès); specimens from the Mediterranean and Atlantic coasts of Spain and Portugal, including the Canary Islands; part of the specimens from the Venice Lagoon.

MOTU-IV: specimens from the Adriatic Sea and a few localities in the Ionian Sea.

In the eight-MOTUs scenario, supported by all three methods (ABGD, GMYC, TCS), the eight major lineages maintained a geographical coherence, and only MOTU-A corresponded exactly to MOTU-I.

We cannot decide with the present data between the two scenarios (four-MOTUs or eight-MOTUs). Pending the availability of more reliable and congruent data from the nuclear genes to support such a decision, we discuss the geographical patterns in the *O. edwardsii* complex focusing on the more detailed (albeit admittedly less conservative) eight-MOTUs scenario.

In the eight-MOTUs scenario, all the specimens attributed preliminarily to *O. edwardsii* were distributed among seven MOTUs: clade B, including specimens from localities in the Aegean Sea; clade C from the Ionian Sea, southwestern Sicily and Lampedusa Island; clade D2 from the Atlantic coast of Spain and Portugal, the Canary Islands and Málaga Province; clade E from several localities across the Tyrrhenian Sea; clade F from Pellestrina, a locality within the Venice Lagoon; clade G from Campania (Pozzuoli and Palinuro); and clade H from the Adriatic Sea and a few localities in the Ionian Sea.

Of these groups, we provisionally identify MOTU-E as representing the 'true' *O. edwardsii*, because it included the specimens sampled in Corsica, its type locality (Payraudeau, 1826). Among our samples, this group is apparently restricted to the Tyrrhenian Sea (eastern Corsica, eastern Sardinia, northern Sicily and western Italian coast: Fig. 4).

The sister lineage to clade E is composed of the D1 and D2 lineages (clade D). The D2 lineage included specimens morphologically ascribed to *O. edwardsii* sampled along the Atlantic coasts of Spain and Portugal, from the Canary Islands and from Málaga Province; the specimens ascribed to *O. leukos* from the Canary Islands; and specimens from populations referred in the recent literature to *O. [cf.] nicolai* from Galicia (Rolán, 1983) (samples 963, 965, 1033, 1058, 1059, 1061–1063), from Barbate (sample 1282) (Gofas, 2011) and from off Ponta da Piedade–Algarve (sample 1294) (Afonso *et al.*, 2011) (Fig. 4). The specimens of *O. edwardsii* sampled along the Atlantic coast of Spain and Portugal displayed a rather distinctive shell morphology with respect to the most common forms found in the Mediterranean.

Ocinebrina leukos was described from Lanzarote (Canary Islands) as living sympatrically with, yet morphologically easily distinguished from, local *O. edwardsii* (Houart, 2000). The latter, however, has been recorded as highly variable morphologically, even in the same locality (Houart, 2000), and, according to our molecular data, *O. leukos* may fall within the variability of this lineage (D2). *Ocinebrina nicolai* was originally described from Corsica, Sardinia and Lipari Islands (Monterosato, 1884). Specimens from the Monterosato collection (see Settepassi, 1977: 38–44), however, are morphologically different from those figured recently under this name (e.g. Rolán, 1983; Houart, 2001; Afonso *et al.*, 2011; Gofas, 2011). Therefore, the association of the name *O. nicolai* with one of our MOTUs is postponed pending the genetic analysis of topotypical specimens.

According to the ABGD and GMYC analyses, clade D also included the specimens from Gabès ascribed to *O. hispidula*, although a distinct well-supported clade (D1) for this lineage has been recovered in all the topologies. The large genetic distance between specimens identified as *O. hispidula* from Gabès and specimens classified preliminarily as *O. edwardsii* from Sicily (clade C) has been reported recently by Barco *et al.* (2013). *Ocinebrina hispidula* has also been reported from other Atlantic and Mediterranean localities, such as Mogador (Ar dovini & Cossignani, 2004), Sicily (Settepassi, 1977; Giannuzzi-Savelli *et al.*, 2003), Cyprus (Houart, 2001; Öztürk, Buzzurro & Benli, 2004) and the Balears (Pons-Moyà & Pons, 2002). Although these records may have been based on extremely spiny morphotypes of other taxa of the *O. edwardsii* complex, or on mislabelled specimens, according to our analyses (ABGD and BEAST-GMYC), *O. hispidula* is included in a more widely distributed MOTU (clade D). Even in the less conservative eight-MOTUs scenario, MOTU-D probably represents a single species ranging from the northeastern Atlantic to the western and central Mediterranean Sea, which has recently colonized the Gulf of Gabès with a population displaying a strikingly deviating morphology (Fig. 4), a phenomenon well known in the Gabès molluscan fauna (Cecalupo *et al.*, 2008), and with a distribution compatible with that of other intertidal molluscs (Calvo *et al.*, 2009). Furthermore, the young age of the Gulf of Gabès (Burolet, Clairefond & Winnock, 1979; Stocchi, Colleoni & Spada, 2009) would not be compatible with the recent evolution of an endemic species. *Ocinebrina erronea* Cecalupo, Buzzurro & Mariani, 2008 (see Table 2 for nomenclatural details) has been synonymized recently with *O. hispidula* by Cossignani & Ar dovini (2011). A single specimen of the *O. erronea* morphotype (sample 1053) was included in our dataset and, in all the analyses, it fell

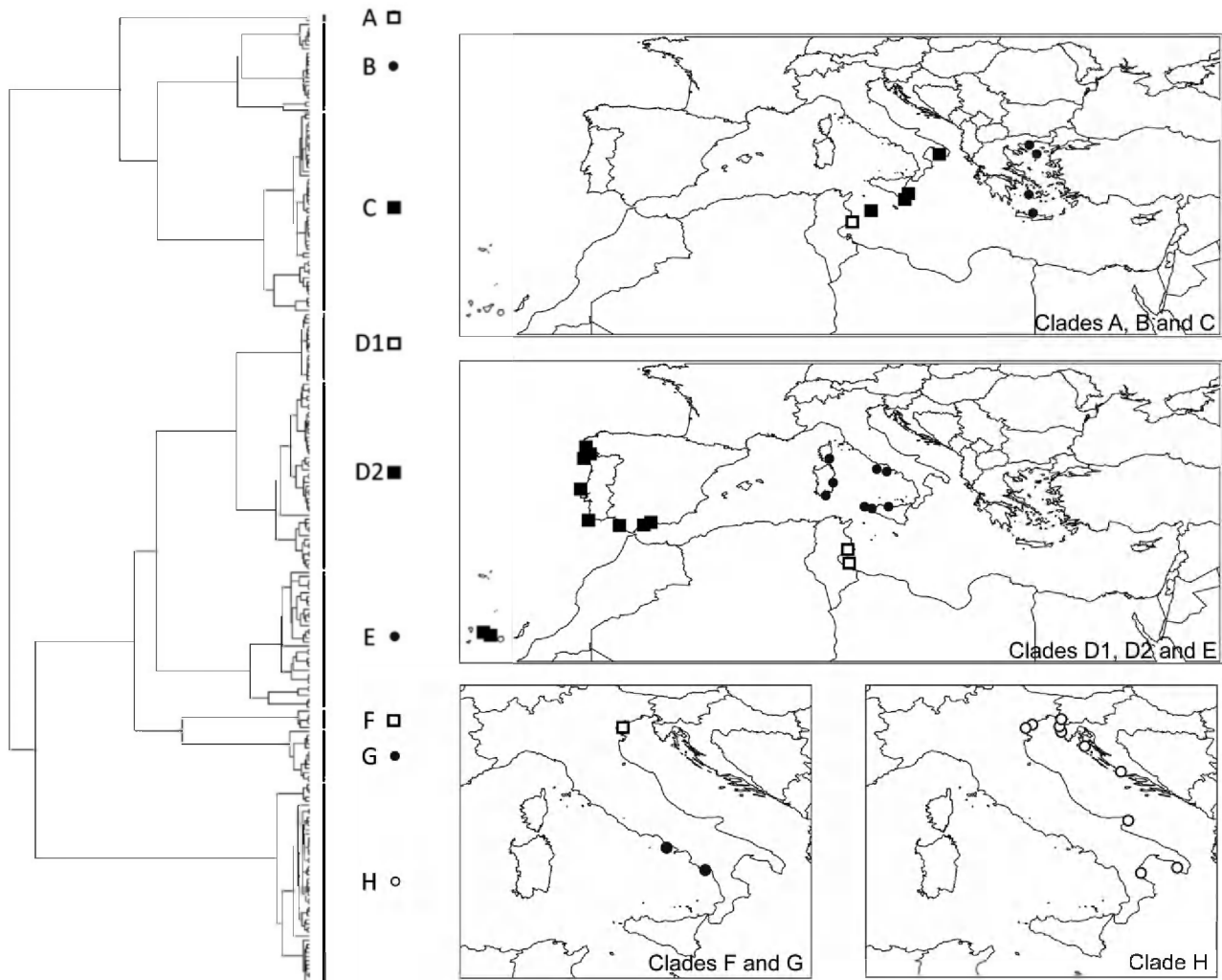


Figure 4. Same tree as in Figure 3 obtained by BEAST on the cytochrome oxidase subunit I (COI) alignment, and distribution maps of the vouchers, grouped in the clades resulting from the COI analysis.

within the *O. hispidula* clade (group D1), indicating that it is not a distinct species.

Ocinebrina piantonii and *O. carmelae* were described as new species from the Kerkennah Islands by Cecalupo *et al.* (2008), living sympatrically (and syntopically) amidst the rhizomes of *Posidonia oceanica* (L.) Delile, 1813. The morphological differences between the two nominal taxa are indeed very subtle, possibly ending up as just two colour forms: a white phenotype (*O. piantonii*) and a dark phenotype (*O. carmelae*). Furthermore, the latter is indistinguishable from *O. hybrida* (Aradas & Benoît, 1876) (which is possibly a synonym of *O. baetica* (Reeve, 1845): see Table 2), a rare species for which we had no specimens for DNA extraction. We assayed two specimens from the Gulf of Gabès, representing the two morphotypes, which proved clearly to belong to a single species (clade A), not related

phylogenetically to *O. hispidula*. They fall into an eastern Mediterranean group including clades B (Aegean Sea) and C (Ionian Sea).

Specimens collected at four localities across the Aegean Sea (clade B) were indistinguishable morphologically from those classified preliminarily as *O. edwardsii* from other Mediterranean localities. The COI and 16S sequences of these specimens were particularly divergent from all the others, allowing the identification of a well-distinct mitochondrial lineage. Furthermore, the mitochondrial data also suggest a well-defined population structure within the group, with three deeply separated clusters corresponding to distinct sampling localities (northern, central and southern Aegean Sea) (Fig. 4).

Another clade (C) is represented by the specimens collected in the Ionian Sea, southwestern Sicily and Lampedusa Island. Similar to group B, clade C

is well defined geographically (Fig. 4), but the shells are rather indistinguishable from specimens ascribed morphologically to *O. edwardsii* from other Mediterranean localities. The sister group relationship with the Aegean clade suggests an allopatric origin of both lineages from an ancestral eastern Mediterranean clade (see below).

Ocinebrina ingloria was described originally by Crosse (1865) without specifying any locality. Other names may apply to this entity (e.g. *Murex orcomenus* Nardo, 1847, see Table 2), but the assessment of the status was beyond the scope of this study. Neglected for over a century, it was recorded as *O. ingloria* recently from the Adriatic Sea, from Grado and the Venice Lagoon (which was interpreted as the most likely locality of the type material) by Houart (2001) and from Palermo by Giannuzzi-Savelli *et al.* (2003). The population at Grado seems to be extinct, together with other intertidal molluscan species, as a result of the intense human activity in the area (see Crocetta, 2011), whereas the record from Palermo seems likely to be incorrect because such a morphotype has never been found there (G. Bonomolo, unpublished). We have assayed specimens from the Venice Lagoon (morphologically and geographically representing the nominal taxon *O. ingloria*) which, despite evident morphological differences, were grouped in clade H with other specimens classified morphologically as *O. edwardsii* from the Adriatic Sea and a few localities in the Ionian Sea (Fig. 4). Apparently all specimens of the *O. edwardsii* complex from the Adriatic Sea belong in the same lineage as *O. ingloria*, again suggesting that shell variation, as currently approached within this group, is rather misleading, and that a single species is involved in this lineage.

The sole geographical exception to clade H is represented by some specimens sampled at Pellestrina (clade F), within the area of the Venice Lagoon. All specimens from this site were indistinguishable from each other morphologically (see, for example, vouchers 1046_1 and 1046_3; Fig 3B, Supporting Information Fig. S4). After the first analyses split the first lot of specimens from this locality (sample 1046) into groups F and H, we collected other specimens from the same locality (sample 1293) in order to rule out the possibility of contamination, and obtained the same results. Thus, according to this evidence, two morphologically indistinguishable MOTUs live sympatrically at this site in the Venice Lagoon. The sister clade to MOTU-F from Pellestrina is clade G from Pozzuoli and Palinuro (central Tyrrhenian Sea). Clades F and G were clearly distinct MOTUs in all of our species delimitation analyses, and both lineages possibly represent relict populations isolated on the two sides of the Italian peninsula.

BIOGEOGRAPHICAL PATTERNS

Comparing our molecular data with the fossil record of *Ocinebrina* would allow the drawing of a time-calibrated tree and a comparison between the estimated origin of clades and the history of the Mediterranean. Unfortunately, although fossil *Ocinebrina* shells are relatively abundant, their species classification based on shell morphology would be rather misleading. What has emerged from the present study is that shell characters used in the taxonomy of *O. edwardsii* and related species do not always define monophyletic lineages, and we have therefore no basis for using fossils to calibrate node ages in the phylogeny. Such an analysis may become possible only after a thorough revision of shell characters used in this group. A comparison with previous studies, however, might be helpful in understanding the observed geographical distribution of molecular lineages.

The present-day Mediterranean marine fauna is the result of a long and troubled geological history (Taviani, 2002) peaking in the Messinian Salinity Crisis (MSC) (Krijgsman *et al.*, 1999) and the following Pliocene and Quaternary glacial/interglacial cycles. These events contributed to the shaping of the current fauna into defined biogeographical categories (Bianchi & Morri, 2000), and the geographical structure among populations of Mediterranean organisms can generally be traced back to these major events (Patarnello, Volckaert & Castilho, 2007). Species with short or no planktonic larval stage (such as the north-eastern Atlantic *Ocinebrina* species) are supposed to show a more structured distribution across a reduced geographical range (Cunha *et al.*, 2005, 2008; Meyer *et al.*, 2005; Paulay & Meyer, 2006) than those with planktonic larvae. Thus, in the Mediterranean Sea, the phylogeography of marine organisms, and especially of those with reduced planktonic stages, is expected to reflect these events. This is the case for reef-building gastropods of the *Dendropoma petraeum* (Monterosato, 1884) complex, which has been shown to include four cryptic species with a clear east–west Mediterranean subdivision (Calvo *et al.*, 2009). This geographical structure has been attributed to vicariant cladogenesis favoured by the reduced planktonic larval stage of the species. We observed a high similarity between the geographical distributions of the lineages of *Ocinebrina* and that found by Calvo *et al.* (2009) in the *D. petraeum* complex.

Our group D has a distribution similar to that of the western Mediterranean *Dendropoma* clade. The continuous range across the Atlanto-Mediterranean border found in many marine species is generally explained as a re-colonization process completed by propagules from extant Atlantic populations

establishing new populations in the Mediterranean. However, restricted gene flow through the Straits of Gibraltar has also been detected in species with high dispersal potential (Sá-Pinto *et al.*, 2012). In species with a short-lived planktonic stage, the dispersal across long distances possibly has been accomplished by rafting or with floating debris (Martel & Chia, 1991; Cunningham & Collins, 1998). The combination of these factors and the effect of the Algerian current flowing along the northern coast of Africa could explain the presence of this lineage in the Gulf of Gabès and its genetic distinctiveness.

The repeated instances of separation of the western and eastern Mediterranean basins by the Sicilian sill during the Quaternary glacial cycles are often correlated with the present-day distinction between corresponding lineages (e.g. Oliverio, 1994, 1996; Calvo *et al.*, 2009; Sá-Pinto *et al.*, 2010; Mejri *et al.*, 2011). We found that three of our groups (MOTU-A, MOTU-B and MOTU-C) formed a well-supported clade (MOTU-I + MOTU-II) in both the BEAST and MrBayes analyses, supporting the existence of an eastern Mediterranean lineage. In the three-gene Bayesian analysis (Fig. S3B), the Adriatic clade (all the Adriatic specimens with the exception of group F) is closely related to groups A, B and C, suggesting an early separation of the ancestor of these four groups from a western Mediterranean clade ancestor.

The phylogenetic relationships of groups F and G with the others are still unclear, making it difficult to draw their position in this scenario. According to BEAST analysis (Fig. 2), both lineages are closely related to the western Mediterranean group (clades D and E), but, in other analyses, support for such a position is lower. The long branches of these groups and their sympatry with other clades (F with H in the Adriatic Sea and G with E in the Tyrrhenian Sea) would suggest early allopatric isolation followed by secondary contact. The actual distribution of these two groups, however, is still unknown, and further studies with denser sampling are required to understand their status.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Result of the Generalized Mixed Yule-Coalescent (GMYC) analysis for the combined COI + ITS2 dataset.

Figure S2. Results of the single-gene Bayesian analyses (by MrBayes). From left to right: COI, ITS2, 16S.

Figure S3. Results of the multiple-gene Bayesian analyses (by MrBayes). From left to right: COI + ITS2 and COI + ITS2 + 16S.

Figure S4. Shells of representative voucher specimens from each sampling site. ID numbers as in Table 1. Scale bar, 10 mm.

Figure S5. Shells of representative voucher specimens from each sampling site. ID numbers as in Table 1. Scale bar, 10 mm.

Figure S6. Shells of representative voucher specimens from each sampling site. ID numbers as in Table 1. Scale bar, 10 mm.

Table S1. Sequence Accession Numbers of the sequences of *Ocinebrina* spp. used in this work, within the framework of the BOLD Project 'BOCI' (Barcoding of *Ocinebrina*).