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Molecular taxonomy in 2D: a novel ITS2 rRNA sequence-structure approach guides the description of the oysters' subfamily Saccostreinae and the genus *Magallana* (Bivalvia: Ostreidae)

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Molecular approaches have contributed to a drastic reconsideration of organisms' systematics and evolution especially in some groups of bivalves where high levels of phenotypic plasticity have hampered morphology-based assessments. However, these insights have rarely been integrated into taxonomy and classification due to the challenge of taxon description based on DNA data alone. In this study we used, for the first time, an approach based on ITS2 rRNA sequence-structure for the diagnosis and description of new oyster taxa identified based on multi-locus phylogenetic analyses including new molecular data for *Striostrea* Vialov, 1936. Phylogenetic analyses and diagnostic ITS2 sequence-structure characters supported Ostreidae Rafinesque, 1815 with four subfamilies: Crassostreinae Scarlato & Starobogatov, 1979 (including *Crassostrea* Sacco, 1897, *Talonostrea* Li & Qi, 1994, and ***Magallana* gen. nov.**), **Saccostreinae subfam. nov.** (including *Saccostrea* Dollfus & Dautzenberg, 1920), Striostreinae Harry, 1985 (including *Striostrea*) and Ostreinae Rafinesque, 1815 (including the remaining genera). We provide a formal description of the subfamily **Saccostreinae subfam. nov**. and the genus ***Magallana* gen. nov.**, a diagnosis for the subfamily Striostreinae, and suggest the reclassification of *Striostrea circumpicta* (Pilsbry, 1904) and *Crassostrea zhanjiangensis* Wu, Xiao & Yu, 2013 as *Ostrea circumpicta* and ***Talonostrea zhanjiangensis* comb. nov**., respectively. This study demonstrates the suitability of ITS2 sequence-structure characters for taxonomic diagnosis and description. The advantages of such an approach in the context of DNA taxonomy of cryptic taxa are discussed.

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INTRODUCTION

The morphology of organisms has been used for identification and classification since before the advent of Linnaean modern taxonomy. Today, taxonomic practice is ideally based on an integrative approach combining morphology with additional information such as molecular data, behaviour, ecology and biogeography (Padial *et al.*, 2010). In the case of cryptic species, which are by definition morphologically indistinguishable, molecular data are often the primary source of information for species identification (DNA barcoding in its strict sense) and species discovery (e.g. Hebert *et al.*, 2003, 2004; Fontaneto *et al.*, 2009; Leasi & Norenburg, 2014). DNA sequence data in the taxonomic context of cryptic lineages may offer a manyfold tool allowing the definition of a phylogenetic (i.e. evolutionary) framework for classification (e.g. Talavera *et al.*, 2012; Salvi, Macali & Mariottini,

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2014), for species delimitation and discovery (e.g. Monaghan *et al.*, 2005; Pons *et al.*, 2006), as well as providing molecular characters for species diagnosis and description (e.g. Halt *et al.*, 2009; Cook *et al.*, 2010).

Species delimitation and discovery is the first step of DNA taxonomy, and is best fulfilled using a cross-validation approach between different methods of species delimitation – based on characters, genetic distance, phylogenetic trees or models (see Goldstein & DeSalle, 2011). Despite early claims by radical DNA taxonomists (Tautz *et al.*, 2003), the need of a formal description and integration in the Linnaean system of these molecularly discovered entities such as molecular operational taxonomic units (MOTUs), OTUs and candidate species is now recognized (Hebert & Gregory, 2005; Goldstein & DeSalle, 2011; Jörger & Schrödl, 2013). However, these final steps of molecular taxonomy, i.e. the taxon description, frequently remain incomplete due to the intrinsic challenge of using DNA sequence information to provide diagnostic characters for taxonomic description (Brower, 2010; Cook *et al.*, 2010; Jörger & Schrödl, 2013).

Molecular phylogenetics and molecular taxonomy have traditionally focused on analysing the primary DNA sequence and these data have been used in several ways in taxonomic descriptions (reviewed by Goldstein & DeSalle, 2011). However, in the last decade several studies have demonstrated the utility of secondary structure information of ribosomal RNA (rRNA) molecules such as the nuclear ribosomal internal transcribed spacer 2 (ITS2) in phylogenetic reconstruction (e.g. Schultz & Wolf, 2009; Keller *et al.*, 2010; Salvi *et al.*, 2010), as well as in diagnosis of species (Müller *et al.*, 2007; Coleman, 2009) and higher taxa (Coleman, 2003; Keller *et al.*, 2008; Salvi *et al.*, 2010, 2014; Salvi & Mariottini, 2012). The ITS2 region of nuclear DNA provides a powerful tool for molecular systematics of eukaryotes because its secondary structure is evolutionarily conserved – as it is of importance in ribogenesis – while its primary sequence shows a relatively high nucleotide substitution rate (Coleman, 2003, 2007; Schultz *et al.*, 2005; Wolf *et al.*, 2005; Müller *et al.*, 2007). This allows the simultaneous sequence-structure alignment not only across multiple species and genera but also at broader taxonomic levels (Coleman, 2003; Seibel *et al.*, 2006).

The ITS2 secondary structure is generally organized in four main helix domains, DI–IV, of which DIII and DIV usually show high sequence variation (Oliverio, Cervelli & Mariottini, 2002; Coleman, 2003, 2007; Schultz *et al.*, 2005; Salvi *et al.*, 2010). These structural domains are supported by minimum free energy and compensatory base changes (CBCs), i.e. mutations in both nucleotides of a paired position in a double-stranded structure of the transcribed RNA. Indeed, the structural conformation having low free energy ensures thermodynamic stability and during evolution the paired nucleotide bonds of each domain are maintained by (compensatory) base changes in both nucleotides of a paired structural position. CBCs in conserved regions of the eukaryote ITS2 sequence-structure have been shown to correlate with interbreeding incompatibility between species, so that the presence of at least one CBC is a good indication (reliability higher than 90%) of two organisms belonging to distinct species (Coleman, 2000, 2003, 2009; Müller *et al.*, 2007). Recent studies on different metazoan groups, and especially on bivalves, have pointed out conserved features of the ITS2 secondary structure such as stem-loop domains that are diagnostic at high taxonomic levels such as tribes, subfamilies, families and orders (Oliverio *et al.*, 2002; Bologna *et al.*, 2008; Keller *et al.*, 2008; Salvi *et al.*, 2010, 2014; Salvi & Mariottini, 2012). Thus, information from ITS2 folding and sequence-structure variation may provide a useful tool in molecular taxonomy for species delimitation and higher-taxon diagnosis. However, while the ITS2 sequence-structure approach has been used to identify diagnostic features of existing taxa, to our knowledge it has never been exploited for the description of new taxa. The fruitful application of such an approach at many taxonomic levels in bivalves (see e.g. Salvi & Mariottini, 2012) allowed us to envisage its great potential for molecular description of taxa.

In the present study we applied, for the first time, an ITS2 rRNA sequence-structure approach for the taxonomic description of a new subfamily and a new genus of oysters, for clades that were uncovered as distinct molecular lineages in a multilocus phylogeny of the bivalve family Ostreidae Rafinesque, 1815 (Salvi *et al.*, 2014). These taxa were given new names therein – Saccostreinae subfam. nov. and *Magallana* gen. nov., respectively – but without a formal description. Thus, these names are currently not available according to the ICZN [International Commission on Zoological Nomenclature] (1999) (Marshall, 2015a,b). Moreover, we generated additional molecular data for the type species of *Striostrea* Vialov, 1936 to assess its classification and provide a molecular diagnosis of this genus. Bivalves such as oysters provide an excellent case study for molecular taxonomy for two main reasons. First, cryptic speciation is a pervasive phenomenon in this group. Both shell and soft-part morphology is often uninformative and shared by different species as it is mostly shaped by phenotypic plasticity rather than by shared evolutionary history (Harry, 1985; Seilacher, Matyla & Wierzbowski, 1985; Huber, 2010; Liu *et al.*, 2011). Second, even at higher taxonomic levels, such as genera and subfamilies, diagnostic

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morphological characters are not robust and traits such as chomata, hyote spines and labial palps have been revealed to be of little taxonomic value (e.g. Littlewood, 1994; Ò Foighil & Taylor, 2000; Salvi *et al.*, 2014). The main aims of this study are: (1) to provide a multilocus phylogenetic framework for oyster systematics and classification using extended taxon sampling compared with Salvi *et al.* (2014); (2) to use the ITS2 rRNA sequence-structure approach as a backbone for the description of newly identified taxa; and (3) to discuss the advantages of using this novel approach in DNA taxonomy of cryptic taxa.

METHODS

Secondary structure modelling

The ITS2 secondary structures were obtained by contrasting several candidate low free energy folding models calculated using RNA structure 5.5 (Reuter & Mathews, 2010) against secondary structure models proposed for molluscs in previous studies (Lydeard *et al.*, 2000; Oliverio *et al.*, 2002; Salvi *et al.*, 2010; Salvi & Mariottini, 2012). Individual secondary structures were used to produce a multiple ITS2 sequence alignment as well as to pinpoint diagnostic characters of oyster taxa.

Data collection and phylogenetic analyses

Genomic DNA from three specimens of the type species of the genus *Striostrea*, *S. margaritacea* (Lamarck, 1819), from Witsand (South Africa) was extracted from dissected foots by using the DNeasy Blood and Tissue Kit (Qiagen). Polymerase chain reaction amplifications of the mitochondrial 16S rRNA (16S) and cytochrome oxidase I (COI) and the nuclear ribosomal ITS2 gene fragments were performed using primers and protocols described in previous studies [16S and ITS2: Salvi *et al.* (2010); COI: Crocetta *et al.* (2015)]. Sequences from the same fragments plus the nuclear 28S rRNA (28S) fragment of 46 ostreid species and the Gryphaeidae *Hyotissa hyotis* (Linnaeus, 1758) and *Neopycnodonte cochlear* (Poli, 1795) were used in the molecular analyses (see Table 1 for details).

Multiple sequence alignment of the 16S, COI and 28S dataset was performed using ClustalX 2.0 (Larkin *et al.*, 2007). ITS2 multiple sequence alignments were performed while simultaneously considering the secondary structure of each sequence in 4SALE 1.7 (Seibel *et al.*, 2006, 2008). This software implements the Clustal W algorithm on a sequence-stru cture scoring matrix specific to eukaryotic ITS2. Single gene alignments were then combined to build three concatenated alignments of mitochondrial (16S+COI, mtDNA dataset), nuclear (ITS2+28S, nucDNA dataset) and mitochondrial+nuclear (16S+COI+ITS2+28S; mt-nucDNA dataset) sequences.

Phylogenetic analyses were carried out in TREEFINDER v. October 2011 (Jobb, 2011) using the maximum-likelihood (ML) method and in mrBayes 3.2.2. (Ronquist *et al.*, 2012) using a Bayesian approach (BA). The monophyly of Ostreidae is supported by many studies and the family Gryphaeidae Vialov, 1936 is the sister group of Ostreidae (see e.g. Salvi *et al.*, 2014; Bouchet, 2015). Therefore, we used the gryphaeid taxa *H. hyotis* and *N. cochlear* as outgroups. ML searches were conducted implementing the optimal models of nucleotide substitution selected by TREEFINDER for each gene fragment under the corrected Akaike Information Criterion (16S, ITS2, 28S: GTR+G; COI: HKY+G). We performed Global tree Searches using 100 random start trees generated through equidistant random walks of random nearest-neighbour-interchanges (NNI) starting from the centre trees obtained by simple ML searches. Nodal support was calculated using the parametric bootstrap (BP) with 1000 replicates for the mt-nucDNA tree or the faster bootstrap approximation based on Expected-Likelihood Weights applied to Local Rearrangements of the tree (LR-ELW; Strimmer & Rambaut, 2002; Jobb, 2011) for the mtDNA and nucDNA trees. BA analyses were performed using the same substitution models as for ML analyses and two independent Markov chain Monte Carlo (MCMC) runs of 10 million generations each, sampled every 1000 generations. MCMC chain convergence was verified by average standard deviation of split frequencies values below 0.01 (Ronquist *et al.*, 2012). Posterior probabilities for nodal support (BPP) was assessed on the 7500 trees (75%) sampled after burn-in (25%).

RESULTS AND DISCUSSION

Phylogenetic evidence for a new systematics of Ostreidae

The recent phylogenetic study by Salvi *et al.* (2014) suggested that none of the subfamilies as morphologically conceived was monophyletic, and supported a different systematic arrangement, with Lophinae Vialov, 1936 lumped in Ostreinae Rafinesque, 1815, the genera *Crassostraea* Sacco, 1897 and *Saccostrea* Dollfus & Dautzenberg, 1920 separated in two distinct subfamilies, and with *Striostrea* basically unassigned to any subfamily. This was in line with results from previous molecular studies, based on 28S, 18S, 16S, COI and complete mitochondrial genome data (see references in Salvi *et al.*, 2014).

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Table 1. GenBank accession numbers of the sequences used in this study

Species	16S rRNA	COI	ITS2 rRNA	28S rRNA
Alectryonella plicatula	AF052072 [6]	–	–	AF130999 [8]
Crassostrea brasiliana	FJ478029 [31]	FJ717640 [32]	FJ478044 [31]	–
Crassostrea columbiensis	–	KP455017 [46]	–	KF370358 [45]
Crassostrea corteziensis	EU733651 [28]	–	–	KF370345 [45]
Crassostrea gasar	EF473271 [24]	FJ717611 [32]	FJ544308 [32]	–
Crassostrea rhizophorae	FJ478032 [31]	FJ717613 [32]	FJ478039 [31]	AF137049 [9]
Crassostrea virginica	AY905542 [18]	JX468901 [41]	EU072460 [26]	AF137050 [9]
Cryptostrea permollis	AF052075 [6]	DQ226524 [19]	–	–
Dendostrea crenulifera	KC847121 [44]	KC683511 [43]	–	KC847142 [44]
Dendostrea folium	AF052069 [6]	–	LM993870 [49]	AF137040 [9]
Dendostrea frons	AF052070 [6]	AB084109 [1]	–	AF137039 [9]
*Hyotissa hyotis**	LM993886 [49]	GQ166583 [35]	LM993876 [49]	AF137036 [9]
Lopha cristagalli	AF052066 [6]	AB076908 [1]	–	AF137038 [9]
Magallana ariakensis **com. nov.**	FJ841964 [34]	AF152569 [10]	EU072457 [26]	AF137052 [9]
Magallana belcheri **com. nov.**	AY160758 [15]	AY160755 [15]	–	Z29545 [50]
Magallana bilineata **com. nov.**	NC013997 [34]	NC013997 [34]	–	–
Magallana dianbaiensis **com. nov.**	AB971997 [5]	AB971935 [5]	–	–
Magallana gigas **com. nov.**	FJ478036 [31]	DQ417696 [21]	LM993864 [49]	AB102757 [2]
Magallana hongkongensis **com. nov.**	FJ841963 [34]	AY632556 [17]	GU338879 [36]	AY632552 [17]
Magallana nippona **com. nov.**	HM015198 [37]	AF300616 [11]	FJ356681 [30]	AB110095 [3]
Magallana sikamea **com. nov.**	HQ660968 [38]	EU816025 [29]	–	AY632554 [17]
*Neopycnodonte cochlear**	JF496758 [40]	AB076939 [1]	LM993878 [49]	AF137034 [9]
Ostrea algoensis	AF052062 [6]	–	–	AF137041 [9]
Ostrea angasi	AF052063 [6]	AF112287 [7]	–	AF137046 [9]
Ostrea aupouria	AF052064 [6]	AY376628 [16]	–	–
Ostrea chilensis	AF052065 [6]	AF112285 [7]	–	AF137045 [9]
Ostrea circumpicta	AB898280 [4]	AB898294 [4]	EU072462 [26]	–
Ostrea conchaphila	AF052071 [6]	–	EF035117 [23]	AF137044 [9]
Ostrea denselamellosa	AF052067 [6]	–	FJ356689 [30]	AF137043 [9]
Ostrea edulis	JF274008 [39]	AF540599 [13]	LM993872 [49]	AF137047 [9]
Ostrea equestris	AY376603 [16]	–	–	DQ242465 [19]
Ostrea futamiensis	LC051605 [48]	AB898290 [4]	–	–
Ostrea puelchana	DQ640402 [22]	DQ226518 [19]	–	AF137042 [9]
Ostrea stentina	EU409053 [27]	DQ313181 [20]	–	DQ242464 [19]
Planostrea pestigris	KC847125 [44]	–	–	KC847146 [44]
Saccostrea cucullata	AF458901 [12]	EU816078 [29]	–	AJ344329 [14]
Saccostrea echinata	KC847127 [44]	KC683513 [43]	–	KC847148 [44]
Saccostrea glomerata	AF353101 [12]	EU007482 [25]	–	Z29552 [50]
Saccostrea kegaki	KC847128 [44]	AB076910 [1]	EU072464 [26]	AB102755 [2]
Saccostrea malabonensis	LC005440 [47]	LC005431 [47]	–	–
Saccostrea palmula	FJ768515 [33]	KP455012 [46]	–	–
Saccostrea scyphophilla	LM993882 [49]	HQ661029 [38]	LM993868 [49]	KC847153 [44]
Striostrea margaritacea†	LT220867–69‡	LT220873–75‡	LT220870–72‡	AF137048 [9]
Striostrea prismatica	–	KP455045 [46]	–	KF370419 [45]
Talonostrea talonata	KC847134 [44]	KC683515 [43]	–	KC847154 [44]
Talonostrea zhanjiangensis **com. nov.**	JX899654 [42]	JX899647 [42]	–	–

Numbers in square brackets after GenBank accession numbers refer to publications that generated the cited GenBank data [1] Matsumoto (2003), [2] Xue *et al.* (2012), [3] Haiyan *et al.* (2004), [4] unpublished sequence of Hamaguchi *et al.* (2013), [5] Sekino *et al.* (2015), [6] Jozefowicz & Ò Foighil (1998), [7] Ò Foighil *et al.* (1999), [8] Park & Ò Foighil (2000), [9] Ò Foighil & Taylor (2000), [10] Ò Foighil *et al.* (1998), [11] Lee *et al.* (2000), [12] Lam & Morton (2006), [13] Lam, Morton & Slack-Smith (2003), [14] Hammer (2001), [15] Lam & Morton (2003), [16] Kirkendale *et al.* (2004), [17] Haiyan *et al.* (2004a), Wang, Xus & Guo (2004b), [18] Milbury & Gaffney (2005), [19] unpublished sequence of Shilts & Ò Foighil, [20] Lapegue *et al.* (2006), [21] Cardoso *et al.* (2007), [22] unpublished sequence of Shilts *et al.* (2006), [23]

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Harvey, Hoy & Rodriguez (2009), [24] Varela *et al.* (2007), [25] Reece *et al.* (2008), [26] unpublished sequence of Shilts *et al.* (2007), [27] Dridi *et al.* (2008), [28] Pérez-Enríquez, Ávila & Ibarra (2008), [29] Xia, Yu & Kong (2009), [30] Kim *et al.* (2009), [31] Melo *et al.* (2010), [32] Lazoski *et al.* (2011), [33] Polson *et al.* (2009), [34] Wu *et al.* (2010), [35] Plazzi & Passamonti (2010), [36] Zhang *et al.* (2012), [37] Yu & Li (2011), [38] Liu *et al.* (2011), [39] Danic-Tchaleu *et al.* (2011), [40] Plazzi *et al.* (2011), [41] unpublished sequence of Hinkley & Sarinsky (2012), [42] Wu, Xiao & Yu (2013), [43] Li *et al.* (2013), [44] unpublished sequence of Li *et al.*, [45] Mazón-Suásteguia *et al.* (2016), [46] Pagenkopp Lohan *et al.* (2015), [47] unpublished sequence of Ozawa (2014), [48] unpublished sequence of Hamaguchi *et al.* (2015), [49] Salvi *et al.* (2014), [50] Littlewood (1994)

*Outgroup species.

†Sequences of the 16S, COI and ITS2 gene fragments were obtained from three specimens (voucher no. BAU2643-BAU2645) sampled in Witsand (South Africa) and deposited in the 'Museo Zoologico Università di Roma - Università La Sapienza'.

‡Sequences generated in this study.

Moreover, the genus *Crassostrea* was split and the Asian Pacific species were assigned to a new genus.

The phylogeny presented here is based on a wider taxon sampling compared with that of Salvi *et al.* (2014) and, while supporting the main results of their study, it allows placing in a phylogenetic and systematic context three additional genera *Striostrea*, *Talonostrea* Li & Qi, 1994, *Planostrea* Harry, 1985 and 16 additional species. We will focus on the relationships of these additional taxa and refer to Salvi *et al.* (2014) for additional information on the phylogeny of Ostreidae.

Overall multilocus phylogenies uncover four main evolutionary lineages corresponding to the subfamilies Ostreinae, Crassostreinae Scarlato & Starobogatov, 1979, Saccostreinae subfam. nov., and a new lineage represented by *S. margaritacea* and *Striostrea prismatica* (Gray, 1825) (Fig. 1; Supporting Information File S1). The support for these four main clades is strong in all the phylogenetic analyses based on different markers (mt and nuc) and phylogenetic methods (ML or BA) (Fig. 1). The relationships between these subfamilies are not resolved, with conflicting results coming from different markers (Fig. 1A, B; see also Salvi *et al.*, 2014 for more details).

The phylogenetic position of *Striostrea* has been unclear in previous studies. Based on the 28S rRNA tree, *S. margaritacea* (type species of the genus *Striostrea*) was either related to *Saccostrea* (Ò Foighil & Taylor, 2000) or lay outside the main oyster clades in a basal position (Salvi *et al.*, 2014). Mitochondrial data by Raith *et al.* (2015) suggested that *S. prismatica* is also very divergent from all other oyster clades. *Striostrea circumpicta* (Pilsbry, 1904), which some authors (e.g. Torigoe, 2004) include in the genus *Ostrea* Linnaeus, 1758, grouped with *Saccostrea* and genera of Ostreinae based on ITS2 data, but this grouping did not receive BP/BPP support (Salvi *et al.*, 2014). Therefore, it was unclear whether *S. circumpicta* was a true *Striostrea*, and whether this genus belongs to the same subfamily as *Saccostrea* (Bouchet, 2015) or to a distinct subfamily (Raith *et al.*, 2015). Mitochondrial and nuclear data of *S. margaritacea* and *S. prismatica* used in this study provide strong evidence that this genus represents an independent oyster lineage, which is well differentiated from the *Saccostrea*, Ostreinae and Crassostreinae lineages (Fig. 1). This is in line with recent results by Raith *et al.* (2015) for *S. prismatica* based on mitochondrial data. These authors suggested including this genus in the new [*sic*] subfamily Striostreinae, based on the uncontroversial relationship of *S. prismatica* with the type species *S. margaritacea.* However, the name Striostreini Harry, 1985 (originally used to designate a tribe of Crassostreinae including *Striostrea* and *Saccostrea*) is clearly available, albeit that the original morphological diagnosis for this taxon (Harry, 1985) would not be suitable for the *Striostrea* clade alone. Here, we provide multi-locus evidence including the type species *S. margaritacea* supporting monophyly and subfamily ranking for Striostreinae Harry, 1985; and we provide a molecular diagnosis for this taxon in the section below. New mitochondrial data of *Ostrea circumpicta* Pilsbry, 1904 (currently *S. circumpicta*) analysed in this study provide compelling evidence that this species is nested within the Ostreinae clade (Fig. 1; Supporting Information File S1). While *S. margaritacea* has unique ITS2 sequence-structure characters compared with other oyster subfamilies, *S. circumpicta* has the typical Ostreinae ITS2 landmarks (see Fig. 2 and below). Based on these results, we suggest assigning *Striostrea* to the subfamily Striostreinae Harry, 1985 (originally as Striostreini), *Saccostrea* to Saccostreinae subfam. nov. (see section below) and placing the Japanese *circumpicta* within Ostreinae as *Ostrea circumpicta*. The monotypic subfamilies Striostreinae and Saccostreinae are neither closely related, nor do they share diagnostic molecular characters (Figs 1, 2), so it is neither advisable nor possible to lump them into a single subfamily.

The genus *Talonostrea* is currently assigned to the subfamily Crassostreinae (Bouchet, 2015). Our

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phylogenetic results support a close relationship between *Talonostrea talonata* Li & Qi, 1994 and the Pacific species *Crassostrea zhanjiangensis* Wu, Xiao & Yu (2013). These two species form a well-differentiated clade within Crassostreinae that is sister to the *Magallana* clade (Fig. 1C). Therefore, the Pacific Crassostreinae form a monophyletic lineage comprising the reciprocally monophyletic genera *Magallana* gen. nov. (see section below) and *Talonostrea*. *Crassostrea zhanjiangensis* should be placed within the latter genus as *Talonostrea zhanjiangensis* comb. nov.

The ostreine genus *Planostrea* is placed with strong support within the Ostreinae lineage, although with an unresolved position (Fig. 1). Ostreinae currently includes ten genera of which eight are monotypic (Bouchet, 2015). The polytypic genera, *Ostrea* and *Dendostrea*, are clearly polyphyletic in all the phylogenetic trees (Fig. 1; Supporting Information File S1; see also Salvi *et al.*, 2014). Therefore, a taxonomic revision is needed for these taxa.

In summary, the systematics of Ostreidae that best reflect their evolutionary history, as inferred from molecular data, is based on four subfamilies: Crassostreinae (including *Crassostrea*, *Talonostrea* and *Magallana* gen. nov.), Saccostreinae subfam. nov. (including *Saccostrea*), Striostreinae (including *Striostrea*) and Ostreinae (including the remaining genera). *Striostrea circumpicta* should be transferred back to *Ostrea* as *Ostrea circumpicta* Pilsbry, 1904 and *Crassostrea zhanjiangensis* moved to *Talanostra* as *Talonostrea zhanjiangensis* comb. nov. A full taxonomic revision is needed for the Ostreinae genera *Ostrea* and *Dendostrea* Swainson, 1835.

Description of Saccostreinae subfam. nov. and *Magallana* gen. nov., and diagnosis of Striostreinae Harry, 1985

Molecular diagnoses of the new taxa Saccostreinae subfam. nov. and *Magallana* gen. nov. and for the subfamily Striostreinae Harry, 1985 are based on the ITS2 rRNA sequences-structures and reported herein. The common derived Ostreidae ITS2 rRNA structure is generally organized in four to five stems, and conserved sequence-structure motifs in the ITS2 secondary structures have been described by Salvi *et al.* (2014). In particular, we have shown that domains I and II are very informative regions, always being identifiable in terms of sequence-structure and position within the ITS2 molecules in different bivalve families including Ostreidae, and show specific sequence-structure landmarks (Salvi *et al.*, 2010, 2014; Salvi & Mariottini, 2012). The detailed analyses of domains I and II provide molecular diagnostic characters of the new taxa proposed (as well as for Crassostreinae and Ostreinae) and are discussed in the following section. Detailed information on ITS2 sequence-structure of the analysed oyster taxa are provided in Supporting Information Files S2 and S3 along with annotation of sequence-structure domains and reference to diagnostic CBCs and sequence motifs.

Saccostreinae Salvi & Mariottini **subfam. nov.**

Type genus: Saccostrea Dollfus & Dautzenberg, 1920.

Species included: Saccostrea circumsuta (Gould, 1850); *Saccostrea cucullata* (Born, 1778); *Saccostrea echinata* (Quoy & Gaimard, 1835); *Saccostrea glomerata* (Gould, 1850); *Saccostrea kegaki* Torigoe & Inaba, 1981; *Saccostrea malabonensis* (Faustino, 1932); *Saccostrea palmula* (Carpenter, 1857); *Saccostrea scyphophilla* (Peron & Lesueur, 1807); *Saccostrea spathulata* (Lamarck, 1819); *Saccostrea subtrigona* (G. B. Sowerby II, 1871), following Bouchet (2015).

Description: The three ITS2 sequences from Saccostreinae, i.e. *Saccostrea cucullata* (acc. no. KC747112), *S. kegaki* (acc. no. EU072464) and *S. scyphophylla* (acc. nos. LM993868, LM993869), show five domains (DI–V) and conform to the typical oyster secondary structure. In spite of sharing a common ITS2 secondary structure, the three *Saccostrea* species have CBCs in the basal region of the DI stem and the base pairing differs from all other oysters analysed. In particular, the Saccostreinae quadruplet is 5′-CGGA/UUCG-3′,

Figure 1. Phylogeny of the Ostreidae derived from mitochondrial, 16S rRNA and COI, and nuclear, ITS2 rRNA and 28S rRNA, gene sequence data using *Hyotissa hyotis* and *Neopycnodonte cochlear* (Gryphaeidae) as outgroups. Maximum-likelihood phylogenetic trees based on the combined mitochondrial DNA dataset (16S rRNA + COI) (A), the combined nuclear dataset (ITS2 + 28S rRNAs) (B) and the combined mitochondrial and nuclear DNA dataset (16S rRNA + COI + ITS2 + 28S rRNAs) (C). Bootstrap values over 1000 replicates (≥70) and Bayesian posterior probabilities (≥0.90) are reported above and below the main nodes, respectively. In the phylogenetic trees shown in A and B, cartoons' length represent clade branch lengths but the height of each cartoon is not proportional to the number of taxa in each clade: for a full representation of these trees see Supporting Information File S1. In the phylogenetic tree shown in C, nuclear gene sequences were unavailable for taxa marked with an asterisk (*) and were coded as missing data. Details on GenBank accession numbers are reported in Table 1.

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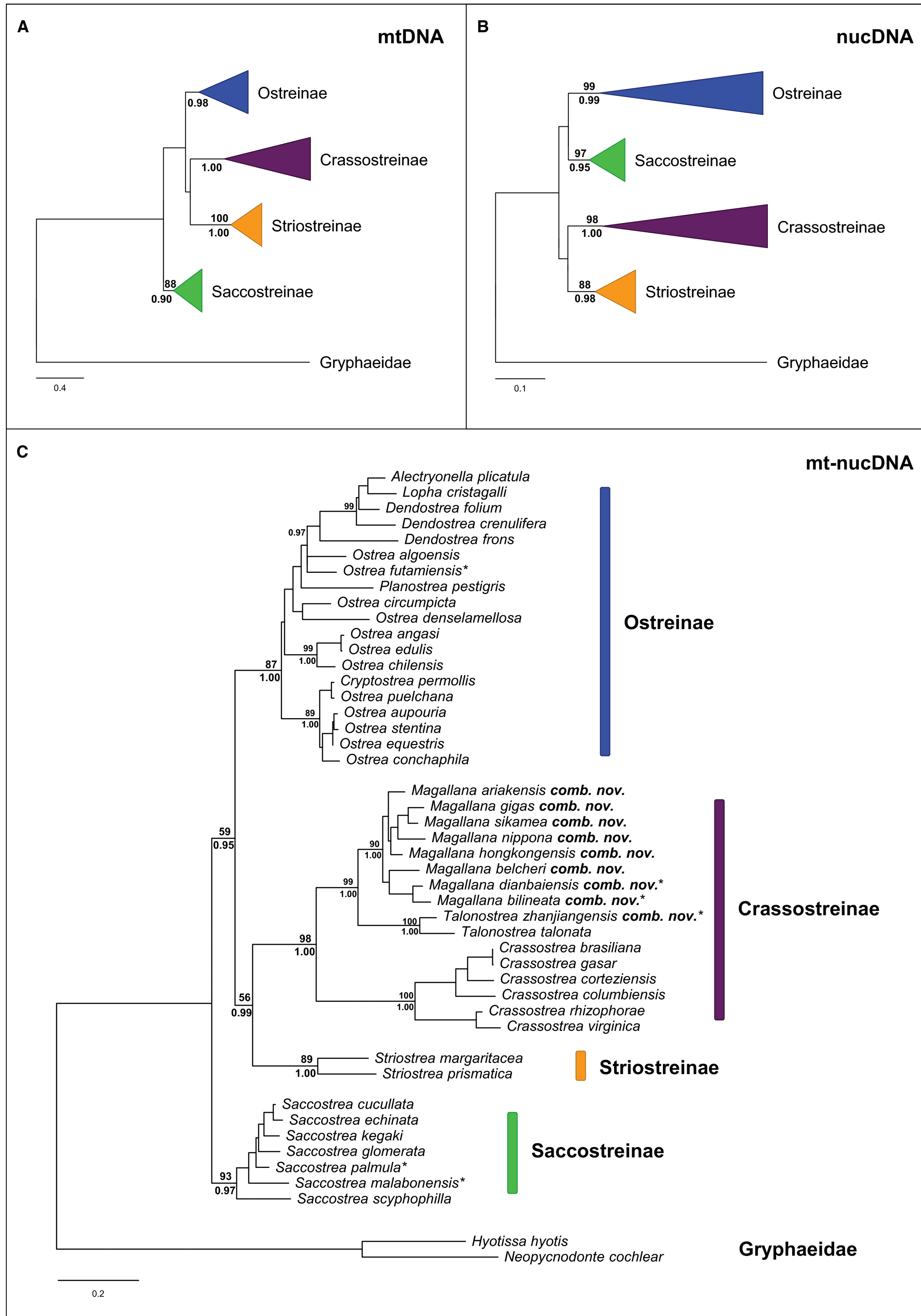

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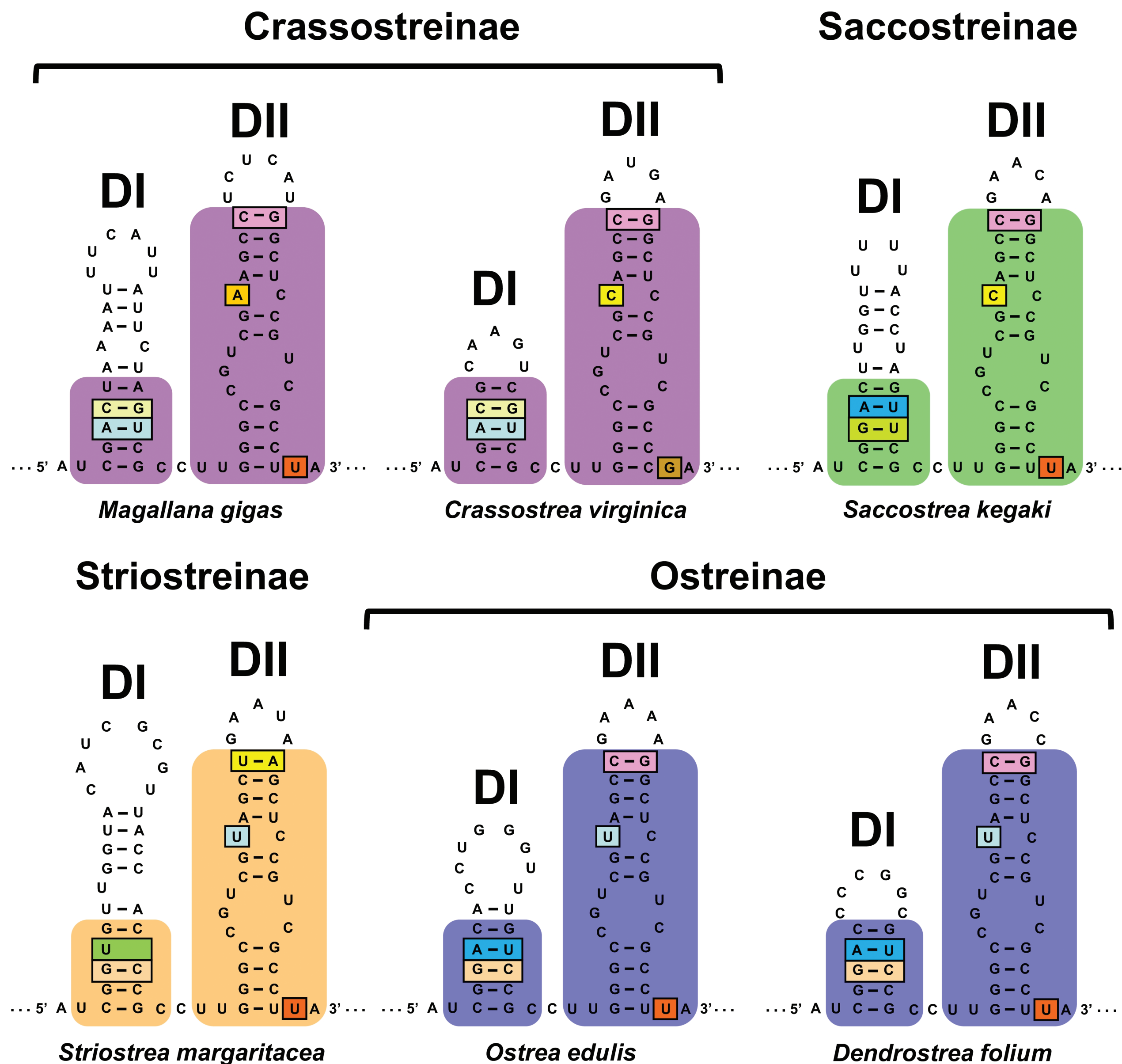

Figure 2. ITS2 secondary structure models of oyster domains I and II. The entire domain DI and DII of each subfamily is boxed with a different colour. Conserved base pairings are boxed with different colours to show subfamilies' diagnostic compensatory changes to be considered as specific sequence-structure landmarks for taxonomic classification. Domain I: yellow and light-blue in Crassostreinae, dark-blue and lime-green in Saccostreinae, ochre and light-green as a single nucleotide in Striostreinae, and dark-blue and ochre in Ostreinae; domain DII: yellow in the upper stem of Striostreinae, pink in Crassostreinae, Saccostreinae and Ostreinae; the single nucleotide in the stem is yellow in *Crassostrea* and ochre in *Magallana* (Crassostreinae), yellow in Saccostreinae, and light-blue in Striostreinae and Ostreinae. Albeit not involved in base pairing, the nucleotide at the 3′ end downstream of the DII stem is boxed in brown in *Crassostrea* (red in all taxa) to show the diagnostic base change occurring in this genus.

showing two CBCs compared to the sequences 5′-CGAC/GUCG-3′ (Crassostreinae), 5′-CGGA/UCCG-3′ (Ostreinae including Lophinae) and 5′-CGGU/–CCG-3′ (Striostreinae), respectively [Fig. 2, see also Salvi *et al.* (2014) and molecular description of *Magallana* gen. nov. below]. Moreover, Saccostreinae members invariantly have the nucleotide cytosine (C) in the conserved single mismatch of DII (also observed in members of the genus *Crassostrea*), which in combination with the nucleotide U located 3′

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downstream of DII provides a further diagnostic landmark of this subfamily.

Striostreinae Harry, **1985**

Type genus Striostrea Vialov, 1936.

Species included: Striostrea margaritacea (Lamarck, 1819); *Striostrea denticulata* (Born, 1778); *Striostrea prismatica* (Gray, 1825); former *Striostrea circumpicta* is transferred into the genus *Ostrea* as *Ostrea circumpicta* Pilsbry, 1904.

Description: The ITS2 sequences from Striostreinae, i.e. *Striostrea margaritacea* (acc. nos. LT220870–72), show five domains (DI–V) and conform to the typical oyster secondary structure. In spite of sharing a common ITS2 secondary structure, Striostreinae's domains I and II show diagnostic landmarks on the conserved domains I and II, consisting of the lack of a base pairing in the DI stem quadruplet 5′-CGGU/–CCG-3′ [see the single nucleotide uracil (U) boxed in Fig. 2] and, more strikingly, a CBC (5′-U/A-3′) in the last base pairing of domain II, which is different from the invariant base pairing 5′-C/G-3′ common to all other Ostreidae sequences so far analysed (Fig. 2).

In conclusion, and also taking into account the diagnostic sequence-structure characters of Saccostreinae pointed out in the previous paragraph, the analysis of highly informative ITS2 sequence-structure of DI and DII, CBCs and conserved motifs allow a distinction among Crassostreinae, Ostreinae, Striostreinae and Saccostreinae subfam. nov., corroborating the phylogenetic distinction of these subfamilies based on mitochondrial and nuclear data (see above).

Magallana Salvi & Mariottini **gen. nov**

Type species: Ostrea gigas Thunberg, 1793; which has been recently re-described by Amaral & Simone (2014) to whom we refer for the shell diagnosis and description.

Species included: Magallana gen. nov. includes the following Asian Pacific species currently (Bouchet, 2015) accepted as: *Crassostrea ariakensis* (Fujita, 1913), *Crassostrea belcheri* (G.B. Sowerby II, 1871) [*Crassostrea gryphoides* (Newton & Smith, 1912) according to Huber (2010) and Amaral & Simone (2014) is a synonym of *Crassostrea belcheri*], *Crassostrea bilineata* (Röding, 1798) [*Crassostrea iredalei* (Faustino, 1932) and *Crassostrea madresensis* (Preston, 1916) according to Huber (2010) and Amaral & Simone (2014) are synonyms of *Crassostrea bilineata*], *Crassostrea dactylena* (Iredale, 1939), *Crassostrea gigas* [*Crassostrea angulata* (Lamarck, 1819) according to Huber (2010) is a synonym of *Crassostrea gigas*], *Crassostrea hongkongensis* Lam & Morton, 2003, *Crassostrea nippona* (Seki, 1934), *Crassostrea rivularis* (Gould, 1861) and *Crassostrea sikamea* (Amemiya, 1928).

Etymology. The name *Magallana* in honour of the Portuguese explorer Fernão de Magalhães (Ferdinand Magellan), who crossed the Pacific Ocean in the first circumnavigation of the Earth.

Description: The Crassostreinae ITS2 rRNA invariantly showed specific sequence-structure landmarks: two CBCs in the basal stem region of the DI and the base pairing differs from all other oyster analysed (see above description). Within this subfamily, the Atlantic genus *Crassostrea* and the Indo-Pacific *Magallana* gen. nov. are diagnosed by two landmarks in the DII of the ITS2 rRNA secondary structure. All the ITS2 rRNA sequences from *Magallana* (represented by *C. ariakensis*, *C. gigas*, *C. hongkongensis* and *C. nippona*) shows invariantly the nucleotide adenine (A) in the conserved single mismatch of DII and the nucleotide U located 3′ next to the lower quadruplet motif of the Basal STEM [see Salvi *et al.* (2014) for the ITS2 secondary structure nomenclature and Fig. 2]. The presence of an A in such a positon within the DII domain is a unique molecular landmark exclusively observed in *Magallana* gen. nov. By contrast, all the ITS2 rRNA sequences from Atlantic *Crassostrea* (represented by *C. brasiliana*, *C. gasar*≡*C. tulipa*, *C. rhizophorae*, *C. virginica*) possess a cytosine (C) (Fig. 2).

Additional molecular features that distinguish these two genera are discussed in Salvi *et al.* (2014) and are briefly mentioned here: (1) *Magallana* species have duplicated mitochondrial genes (trnM, trnK, trnQ and rrnS) compared with *Crassostrea* species and show an unusually high conservation of mitochondrial gene order that is very different from *Crassostrea* species (Ren *et al.*, 2010); and (2) in the nuclear genome, karyological differences in the size and shape of the rDNA-bearing chromosome (the chromosome where the major rRNA genes are located) clearly and consistently divide *Magallana* and *Crassostrea* (see Wang *et al.*, 2004).

Concluding remarks on the use of the ITS2 rRNA sequence structure approach in molecular taxonomy

It is now widely accepted that DNA taxonomy should be integrated into the Linnaean system and that taxon descriptions should be character-based (Hebert & Gregory, 2005; Bauer *et al.*, 2011; Goldstein & DeSalle, 2011). However, there is still no standard procedure for taxonomic descriptions based on molecular data. A significant contribution in this direction has been provided by Jörger & Schrödl (2013). These authors describe a pipeline based on the Character

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Attribute Organization System (CAOS; Sarkar, Planet & Desalle, 2008) to determine diagnostic nucleotides of pre-defined species to be used as taxonomic characters in their description and discussed a best practice in molecular taxonomy. Ideally, this procedure should be based on multiple genes, different alignment algorithms and masking options, and should refer to the alignment position of diagnostic nucleotides in a reference sequence or better in a reference genome. As pointed out by Jörger & Schrödl (2013), the main challenges of this sequence-based approach concern the alignment step and the evaluation of apomorphic (i.e. derived) character states. With the exception of protein coding DNA sequences (where the innate punctuation provided by the triplet code makes the alignment straightforward and stable when new data are added), DNA sequence alignments are anything but consistent across different algorithms, parameters and masking options. Moreover markers such as the 16S and the 28S rRNA that are widely used in molecular systematics can be difficult to align even at low taxonomic level. This implies a labile reference for the position of diagnostic nucleotides and, most importantly, an inherent difficulty in establishing homology among molecular characters especially at high taxonomic levels. Even when character homology is ensured across the alignment, the high chance of convergent substitutions among the four character states (i.e. bases) makes it highly probable that character states are shared because of homoplasy.

These drawbacks are overcome by the sequence-structure approach applied in this study. The high conservation of ITS2 secondary structure across eukaryotes (Coleman, 2003, 2007; Schultz *et al.*, 2005; Wolf *et al.*, 2005), and especially within metazoans, at different taxonomic levels such as orders, families and genera, makes the inference of secondary structure straightforward by means of homology modelling (Wolf *et al.*, 2005; Schultz & Wolf, 2009; Koetschan *et al.*, 2012). Secondary structures provide a backbone of information for sequence alignment, which can be computed while simultaneously accounting for individual ITS2 sequence and structure (Seibel *et al.*, 2006, 2008). Given one or more ITS2 sequences, many tools and a well-established phylogenetic pipeline are available for obtaining secondary structures and multiple sequence-structure alignments based on an ITS2-specific scoring matrix and substitution model calculated from thousands of eukaryote sequences from the ITS2 database [see Schultz & Wolf (2009) for an overview and Salvi *et al.* (2014) for a recent example]. Therefore, a standard procedure with ITS2-specific models can be used to build the ITS2 sequence-structure alignment and this is suitable even for comparisons at high taxonomic level. Instead of providing an alignment, a file such as the one provided in Supporting Information Files S2 and S3 with individual ITS2 sequence-structures, in fasta/Vienna format, including CBCs and domains annotation may allow reproducibility and traceability of molecular diagnostic characters and their easy visualization using tools such as 4SALE (Seibel *et al.*, 2008).

As regards convergent evolution of characters, CBCs provide good insurance against these events. CBCs within conserved sequence-structure motif in the ITS2 secondary structure are rare and unique features likely to be of single (monophyletic) evolutionary origin (Coleman, 2003). This makes them less prone to homoplasy even at high evolutionary distances such as those observed between ingroup and outgroup taxa.

On a less positive note, the ITS2 sequence-structure approach may have some limitations due to the fact that it is based on a single locus. While we can exclude idiosyncrasies of this locus, given the crucial role of this molecule for ribogenesis and thus for cell life, or problems due to paralogues, as the many copies of this gene are stabilized by concerted evolution (Coleman, 2003), we could anticipate a possible lack of diagnostic characters at low taxonomic level such as in comparisons between closely related species. This is probably not the case for many organisms given the high evolutionary rate of the primary sequence within less conserved ITS2 domains, such that ITS2 has proven a powerful tool for universal DNA barcoding of animals, fungi and plants (Chase & Fay, 2009; Bellemain *et al.*, 2010; Yao *et al.*, 2010). Thus, the combined use of this approach with a multilocus approach based on primary sequences such as that described by Jörger & Schrödl (2013) would provide a more robust molecular diagnosis for species description.

Finally, we want to emphasize that either ITS2 sequence-structure characters or specific nucleotides provided by CAOS analyses alone do not tell us anything about the novelty of a taxon and its placement in a systematic and evolutionary context. They are instead useful molecular attributes to describe cryptic taxa that should be previously defined by expert taxonomists based on a taxon-wide multilocus phylogenetic framework and if possible on additional sources of information following an integrative approach (Lipscomb, Platnick & Wheeler, 2003; Sebergemail *et al.*, 2003; DeSalle, Egan & Siddall, 2005; Padial *et al.*, 2010; Goldstein & DeSalle, 2011).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the supporting information tab for this article:

File S1. Maximum-likelihood phylogenetic trees based on the combined mitochondrial DNA dataset (16S rRNA + COI) (A) and the combined nuclear dataset (ITS2 + 28S rRNAs) (B). Bootstrap values (≥70) and Bayesian posterior probabilities (≥0.90) are reported above and below the main nodes, respectively.

File S2. Individual ITS2 sequence-structure of the oyster taxa analysed in this study (plus outgroups) in fasta/Vienna format. GenBank accession numbers and acronyms used in Salvi *et al.* (2014) are provided. The file can be easily visualized using the software 4SALE (Seibel *et al.*, 2008).

File S3. Annotated individual ITS2 sequence-structure of the oyster taxa analysed in this study. GenBank accession numbers are provided. Main domain and diagnostic molecular characters used in taxon descriptions (CBCs and sequence motifs) are marked.

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