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Mitochondrial DNA and morphological identification of a new cupped oyster species *Crassostrea dianbaiensis* (Bivalvia: Ostreidae) in the South China Sea

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Abstract – Though *Crassostrea* oysters have been distributed and cultured worldwide, their taxonomy is still difficult and often inaccurately determined because of the high level of phenotypic plasticity of the shell morphology. With the help of mitochondrial DNA, two novel species of *Crassostrea* oysters (*C. hongkongensis* and *C. zhanjiangensis*) were recently recognized, which suggests that the species diversity of Indo-Pacific oysters could be underestimated. Utilizing a combination of shell characteristics, a molecular marker (mitochondrial *cox1* gene) and phylogenetic analysis, we identified a mangrove-distributed novel *Crassostrea* oyster species, *C. dianbaiensis*. The shell morphology of the new species is phenoplastic, as seen in other congeneric oysters. The left valve of this oyster is usually deeply cupped, and the right valve is usually slightly concave. The body size is classified as medium and is approximately 5–10 cm in height (estimated from 20 individuals). One distinctive feature of *C. dianbaiensis* is that the adductor muscle scars vary in color from dark purple to white in the right valve, but always appear white in the left valve. Based on the *cox1* phylogenetic tree, *C. dianbaiensis* is inferred to be a new member of the Southeast Asia tropical oysters and is believed to be the northernmost distributed species among the tropical oysters. This study provided basal information for future studies, which are necessary to better understand the faunal characteristics, population and roles of this oyster in nearshore ecosystems.

Keywords: *Crassostrea* / *cox1* / new species / oyster / molecular identification

1 Introduction

Oysters (Family Ostreidae), as ecosystem engineers, can provide many ecosystem goods and services by creating habitat used by other species and modifying the physical and chemical environment; these changes can have major consequences for estuarine populations, communities, and food webs (Jackson et al. 2001; Ruesink et al. 2006). Based on the shape of the shell, the oyster is one of the most variable bivalves in the world (Gunter 1950), and therefore, morphology is of limited value for species identification and taxonomy (Reece et al. 2008; de Melo et al. 2010). *Crassostrea* oysters are widespread and the most commercially important group of oysters, and hence have received more attention concerning their taxonomy, phylogeny and population genetics (Lam and Morton 2003; Reece et al. 2008; Liu et al. 2011; Li et al. 2013; Wu et al. 2013). Currently, a total of 35 *Crassostrea* oysters have been confirmed by professional taxonomists and listed in taxonomic databases such as WoRMS (World Register of Marine Species, http://www.marinespecies.org), WMSDB (Worldwide Mollusc Species Data Base, http://www.bagniliggia.it/WMSD/WMSDhome.htm), eoL (Encyclopedia of Life, http://eol.org/) and in Huber (2010); 14 of these species have DNA sequences available in GenBank (Wu et al. 2013). Recently, Wu et al. (2013) reported a new species (*C. zhanjiangensis*) in China based primarily on molecular evidence. This Indo-Pacific oyster, previously assumed to be a juvenile of the Kumamoto oyster (*C. sikamea*) by local farmers, could potentially influence the efficiency of *C. hongkongensis* spat collection due to niche competition on spat collection devices.

The rapid growth of bivalve aquaculture and conservation concerns for marine resources has created a pressing need to better understand the species biodiversity and faunal relationships among oyster species. Mitochondrial DNA sequences have proven useful as molecular markers and have been successfully employed to help in species identification and to resolve taxonomic confusion in *Crassostrea* oysters (Reece et al. 2008). The Hong Kong oyster (*C. hongkongensis*) was the first commercially important taxa to be identified using genetic data (Lam and Morton 2003; Boudry et al. 2003). Subsequently, Wang et al. (2004) used mitochondrial 16S rRNA and *cox1* genes to conclude that *C. rivularis* (the closer-river oyster) is actually a species complex comprised of two species (*C. hongkongensis* and *C. ariakensis*). Similarly, using

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Table 1. Species identification of *Crassostrea* oysters collected from different locations and during the months indicated.

Location	Date	Number of individuals	Species (number; percentage)
Dianbai	December, 2005	101	*C. angulata* (32; 31.7%)
			C. dianbaiensis (8; 7.9%)
			C. hongkongensis (17; 16.8%)
			C. sikamea (44; 43.6%)
Dianbai	April, 2012	69	*C. angulata* (13; 18.8%)
			C. ariakensis (22; 31.9%)
			C. dianbaiensis (0; 0%)
			C. hongkongensis (9; 13.0%)
			C. sikamea (25; 36.2%)
Dianbai	November, 2012	156	*C. angulata* (55; 35.3%)
			C. ariakensis (33; 21.2%)
			C. dianbaiensis (4; 2.6%)
			C. hongkongensis (26; 16.7%)
			C. sikamea (38; 24.4%)
Dianbai	January, 2013	115	*C. angulata* (40; 34.8%)
			C. ariakensis (18; 15.7%)
			C. dianbaiensis (0; 0%)
			C. hongkongensis (25; 21.7%)
			C. sikamea (32; 27.8%)
Qinglan	December, 2005	9	*C. dianbaiensis* (0; 0%)
			C. iredalei (8; 100%)
Qinglan	April, 2012	51	*C. dianbaiensis* (12; 23.5%)
			C. iredalei (39; 76.5%)

mitochondrial DNA sequences, Wang et al. (2010) recommended that the Pacific oyster (*C. gigas*) and the Portuguese oyster (*C. angulata*) should be referred to as two subspecies under the name of *C. gigas* (*C. gigas gigas* and *C. gigas angulate*, respectively). Studies concerning molecular identification, phylogeny and investigations of geographic distribution have been performed for *Crassostrea* oysters throughout the world (Cordes et al. 2008; Liu et al. 2011), in countries such as South America (de Melo et al. 2010), Thailand (Bussarawit 2003), China (Xia et al. 2009; Wu et al. 2010) and Asia (Reece et al. 2008).

Molecular analysis can, on the one hand, determine the geographic distribution pattern of existing species, while on the other hand, provide the opportunity to discover and identify cryptic species that are difficult to identify morphologically but are genetically distinct. For example, de Melo et al. (2010) analyzed 292 oysters and revealed a total of three species of *Crassostrea* (*C. gasar*, *C. rhizophorae* and *C. virginica*) at 16 locations along the Brazilian coast, whereas an unassigned *Crassostrea* sp. from Canela Island was shown to be more similar to Indo-Pacific oysters, and either arrived in the Atlantic Ocean before the convergence of the Isthmus of Panama or was accidentally brought to Brazil by ship. In the study of Liu et al. (2011), a total of five unassigned Indo-Pacific species were identified, including two *Crassostrea* (*C.* sp. 1; *C.* sp. 2), two *Saccostrea* (*S.* sp. 1; *S.* sp. 2) and one *Ostrea* (*O.* sp.). These findings support the notion that the species diversity of Indo-Pacific oysters may have been underestimated (Wu et al. 2013). The unassigned species *Crassostrea* sp. 2 in the study of Liu et al. (2011) might be *C. zhanjiangensis*, a newly described Indo-Pacific oyster, based on the nearly identical 16S rRNA sequences of these two species (Wu et al. 2013). Meanwhile, the phylogenetic status of the unassigned species *Crassostrea* sp. Canela, in the study of de Melo et al. (2010), was inferred as a sister species of *C. zhanjiangensis* (Wu et al. 2013).

The coastal areas along the South China Sea in China are species-rich regions of *Crassostrea* oysters (Xia et al. 2009). At least ten *Crassostrea* oysters have been described based on morphological characters and mtDNA data, including *C. gigas*, *C. angulata*, *C. sikamea*, *C. ariakensis*, *C. hongkongensis*, *C. nippona*, *C. belcheri*, *C. iredalei*, *C. vitrefacta* and *C. zhanjiangensis* (Xia 2008; Wu et al. 2013). This study initiated a series of field investigations of oysters' species diversity along the South China Sea (Xia 2008). We used a partial mitochondrial *cox1* gene as a molecular marker for species identification. A total of 352 individuals from 19 locations were sequenced and classified into ten recognized species in five genera and one unassigned species of *Crassostrea*. The unassigned species was *Crassostrea* sp. DB, which was found in Dianbai County, Guangdong province and Qinglan Harbor, Hainan province, and is genetically distinct from any other known *Crassostrea* species but very close to the slipper-shape oyster *C. iredalei* (Xia 2008; Wu et al. 2012). Therefore, in this study, we aimed to present detailed morphological descriptions of shell characteristics and determine the specific identity of this Dianbai oyster using a molecular marker (mitochondrial *cox1* gene) and a phylogenetic analysis.

2 Materials and methods

2.1 Sample collection

Samples of the unidentified oyster individuals were collected at different times from Shuidong bay (N21°30′, E111°0′), Dianbai County, Guangdong province and Qinglan Harbor (N19°33′, E110°49′), Hainan Province (Table 1).

Table 2. List of the samples and *cox1* sequences from GenBank used in this study.

Species	Locality	GenBank no.
Crassostrea angulata	Taiwan, China	NC012648
Crassostrea ariakensis	Yingkou, Liaoning Province, China	NC012650
Crassostrea belcheri	Surat Thani, Thailand	EU007466
Crassostrea dianbaiensis	Dianbai, Guangdong, China	JQ060958
Crassostrea gasar	Unknown	FJ717611
Crassostrea gigas	Unknown	NC001276
Crassostrea gryphoides	Goa, India	EU007489
Crassostrea hongkongensis	Hainan Province, China	NC011518
Crassostrea iredalei	Wenchang, Hainan Province, China	NC013997
Crassostrea madrasensis	Goa, India	EU007460
Crassostrea nippona	Ogatsu Bay, Japan	NC015248
Crassostrea rhizophorae	Santa Catarina State, Brazil	FJ717638
Crassostrea sikamea	Nantong, Jiangsu Province, China	NC012649
Crassostrea sp. 1	Wenchang, Hainan Province, China	HQ661024
Crassostrea sp. Canela 1	Bragança (Canela Island)	HM003525
Crassostrea sp. Canela 2	Bragança (Furo do Café)	HM003526
Crassostrea virginica	Delaware Bay, USA	NC007175
Crassostrea zhanjiangensis	Zhanjiang, Guangdong, China	JX899646
Saccostrea glomerata	Hobart, Tasmania, Australia	EU007483
Saccostrea mordax	South China Sea	NC013998
Ostrea denselamellosa	Qingdao, Shandong Province, China	NC015231
Ostrea edulis	Unknown	NC016180

2.2 DNA extraction, PCR amplification and sequencing

The mitochondrial DNA of each individual was extracted from the adductor muscle or visceral mass using the TIANamp Marine Animals DNA kit (Tiangen, Beijing). A partial *cox1* segment was amplified by PCR with primer pairs of COIL1490 (5′-GGTCAACAAATCAT AAAGATATTGG-3′) and COIH2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). The PCR reactions were performed in a 25 μl volume with 0.5 μl of template DNA (approximately 30 ng), 0.5 μl of 10 mM dNTP mix, 2.5 μl of 10 × buffer (Mg^{2+} plus), 1 μl of each primer (10 μM), and 0.25 μl (1 U) of Ex*Taq* polymerase (Takara, Dalian, China). The PCR reactions were performed on an ABI Veriti thermal cycler (Applied Biosystems, California, USA) with the following parameters: pre-denaturation at 94° for 1 min, followed by 35 cycles of 94° for 20 s, 52° for 20 s, extension at 72° for 1 min, and a final extension step at 72° for 5 min. The PCR products were separated by electrophoresis on a 1.0% agarose gel, purified with a QIAquick PCR Purification kit (QIAGEN, California, USA) and bi-directionally sequenced on an ABI 3730xl DNA Sequencer (Applied Biosystems, California, USA).

2.3 Molecular identification and phylogenetic analysis

A BLASTn search was performed in GenBank for each *cox1* sequence. To confirm the taxonomic status of the morphologically novel species, *cox1* sequences of the new species and those of other species of *Crassostrea*, *Ostrea* and *Saccostrea* available from GenBank were subjected to phylogenetic analysis (Table 2).

The sequences were aligned using MEGA 5 (Tamura et al. 2012). Maximum parsimony (MP), neighbor-joining (NJ) and maximum likelihood (ML) were employed for the phylogenetic reconstructions. The MP analyses were performed using PAUP 4.0b10 (Swofford 2002), with a total of 1000 random addition searches using tree bisection-reconnection (TBR). The Bootstrap (BP) values were calculated from 1000 bootstrap replicates with 10 random additions per replicate in PAUP. The NJ analyses were performed in MEGA 5, and ML analyses were performed using both MEGA 5 and PhyML 3.0 online execution (http://www.atgc-montpellier.fr/phyml). Two *Ostrea* species and two *Saccostrea* species were employed as multiple outgroups.

2.4 Description of shell characteristics

Conchological distinctions used for the identification of other *Crassostrea* oysters were employed for the description of shell features and characteristics of the new species (Lam and Morton 2003; Qi 2004; Huber 2010), including the shape and surface sculpture, external and internal shell color, attachment area of valves and position and color of the adductor muscle scar.

3 Results

3.1 Molecular identification and phylogeny

A total of 501 oyster specimens (Table 1) were sequenced to identify the oyster species by comparison with sequences available in GenBank (Table 2). Using a BLASTn search, the identified sequences included 140 from *C. angulata*, 73 from *C. ariakensis*, 77 from *C. hongkongensis*, 139 from *C. sikamea* and 48 from *C. iredalei*; the remaining 24 sequences were nearly identical to each other but did not precisely match any sequences from other oyster species in the database. These individuals were therefore identified and designated as a new species, *C. dianbaiensis*.

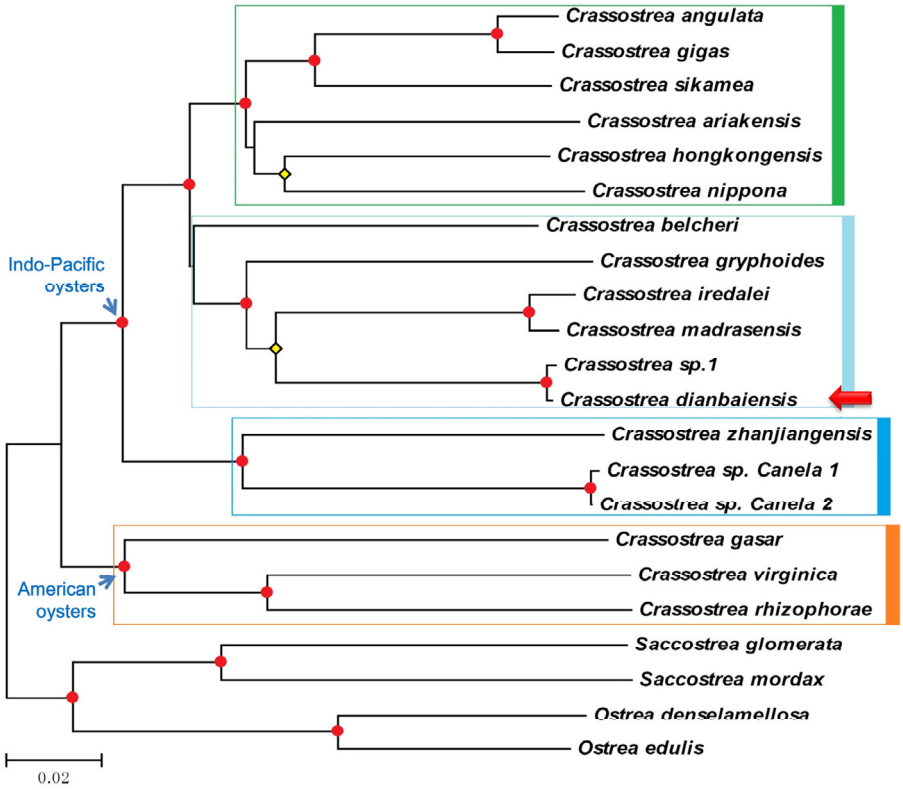

Fig. 1. Phylogenetic reconstruction of *Crassostrea* oysters using partial mitochondrial *cox1* gene sequences. The neighbor-joining (NJ) tree is shown. Bootstrap (BP) values were calculated for maximum likelihood (ML), NJ and maximum parsimony (MP) analyses. Nodes with high statistical support (BP ≥ 75) from all three phylogenetic methods (i.e., NJ, ML and MP) are marked with a solid circle, while moderate supports (50 ≦ BP < 75) are marked with a solid rhombus.

In the phylogenetic tree, *Crassostrea* oysters are resolved as a monophyly group and consist of two lineages: an American oyster lineage and an Indo-Pacific oyster lineage (Fig. 1). Three clades are recognized among the Indo-Pacific oysters: 1) the Zhanjiang oyster (*C. zhanjiangensis*) and the unassigned Brazilian oyster (*Crassostrea* sp. Canela) cluster together as the most basal taxon in the Indo-Pacific oyster lineage; 2) six commonly seen Asian oysters (*C. angulata*, *C. gigas*, *C. sikamea*, *C. ariakensis*, *C. hongkongensis* and *C. nippona*) are grouped together; the species of this second clade are mainly distributed along the Japan Sea, the Southwest coast of the Pacific ocean, the Yellow Sea, the Bohai Sea, the East China Sea and north of the South China Sea; 3) the Dianbai oyster is grouped together with other Southeast Asian oysters (*C. belcheri*, *C. gryphoides*, *C. iredalei* and *C. madrasensis*). The species of this clade are primarily distributed along the coastal waters of the South China Sea, the Gulf of Thailand, the Andaman Sea, the Bay of Bengal and the surrounding regions.

3.2 Species description

Systematics
Order Ostreoida Ferussac, 1822
Suborder Ostreina Ferussac, 1822
Superfamily Ostreoidea Rafinesque, 1815
Family Ostreidae Rafinesque, 1815
Crassostrea dianbaiensis new species

3.2.1 Type measurements and deposition

The holotype specimen and three paratype specimens comprised of dry shells and tissues preserved in 95% ethanol have been deposited in the Marine Biodiversity Collection of the South China Sea, South China Sea Institute of Oceanology, Guangzhou. The holotype and all paratype specimens have been genetically identified. The shell measurements of the type materials are shown in Table 3.

3.2.2 Description of the holotype

The left valve of the holotype of *C. dianbaiensis* is deeply cupped and the right valve is slightly convex (Fig. 2). *C. dianbaiensis* is moderately sized, elongated and orbicular, with a thick shell, and has a surface showing growth squamae on the right valve. The margins of both valves are slightly fluted. The attachment area is small and appears as a patch at the umbone with the rest of the left valve surface unattached. The direction of growth of the left valve is counter to that of the right, producing concentric and slightly erect layers of old ventral margins during each season of fast growth. The umbonal cavity is medium on the left valve. The outer shell surface is chartreuse, while the shell interior is translucent white. The adductor muscle scar is large, located toward the posterior side between the middle and ventral third of the pallial area and is light purple and oval-shaped in the right valve and white in the left valve.

Table 3. Shell measurements of the type materials.

Accession number	Height (mm)	Length (mm)	Depth (mm)	Notes
Holotype SCSMBM 001621	55.8	48.2	28.1	Light purple adductor muscle scar of right valve, white muscle scar of left valve, orbicular shaped
Paratype 1 SCSMBM 001622	53.3	54.0	37.1	Light purple adductor muscle scar of right valve, white muscle scar of left valve, orbicular shaped
Paratype 2 SCSMBM 001623	56.1	47.6	35.2	White adductor muscle scar of both valves, oval shaped
Paratype 3 SCSMBM 001624	54.5	31.8	18.5	Dark purple adductor muscle scar of right valve, white muscle scar of left valve, long-oval shaped

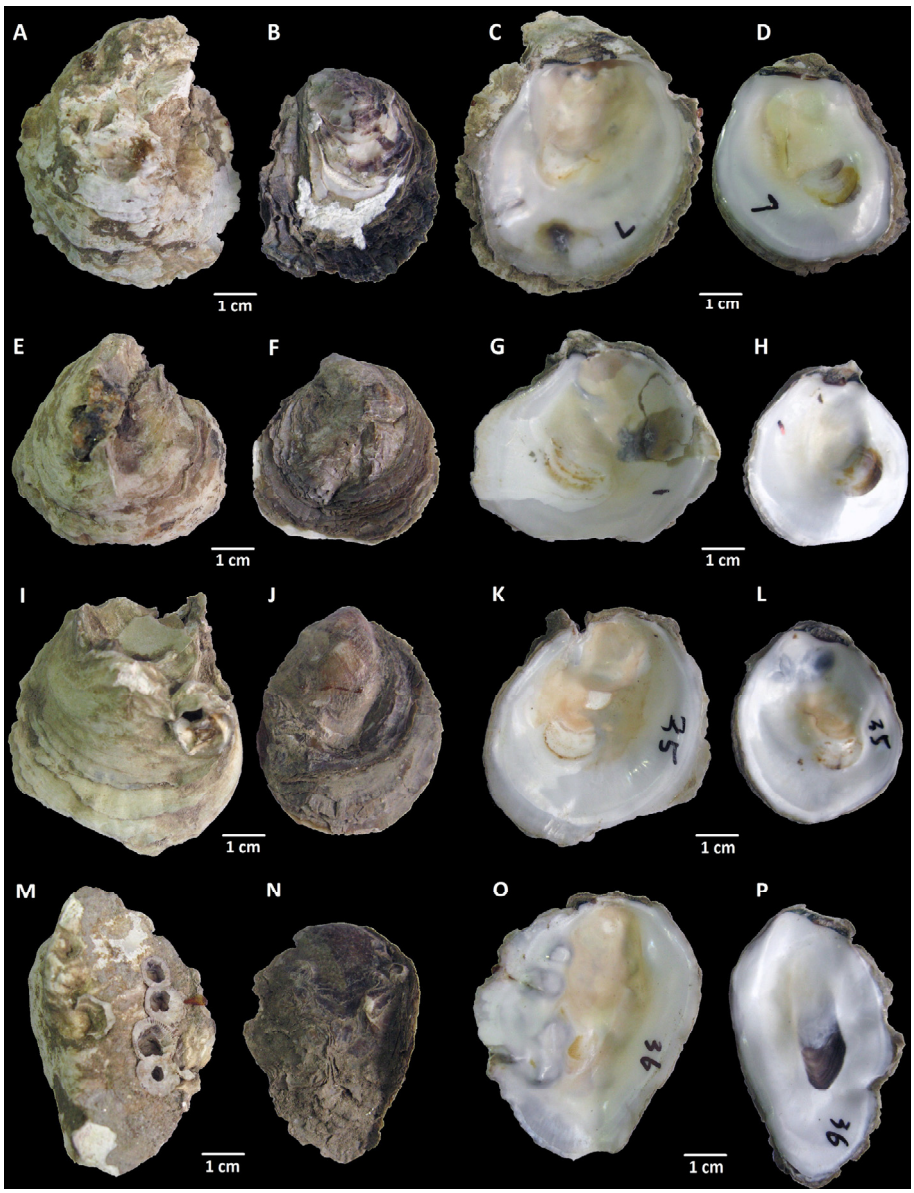

Fig. 2. Shell morphology of *C. dianbaiensis* collected from Dianbai County, Guangdong province. The holotype's external view of the left (A) and right valve (B); the holotype's internal view of the left (C) and right valve (D); external view of the left (E) and right (F) valve of the paratype 1; internal view of the left (G) and right (H) valve of paratype 1; external view of the left (I) and right (J) valve of paratype 2; internal view of the left (K) and right (L) valve of paratype 2; external view of the left (M) and right (N) valve of paratype 3; internal view of the left (O) and right (P) valve of paratype 3.

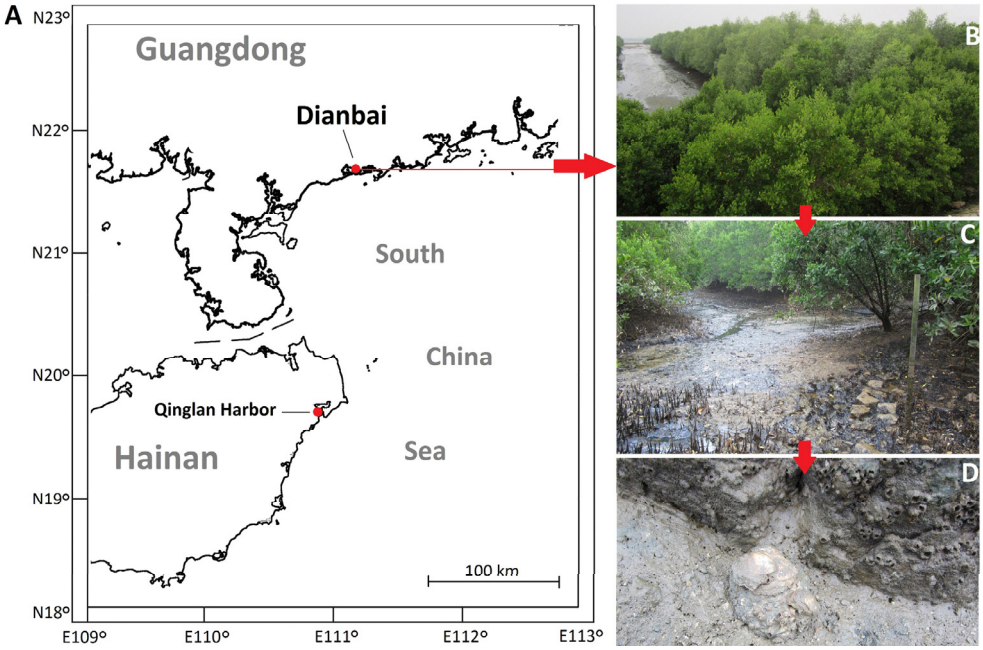

Fig. 3. (A) Two sampling positions of *C. dianbaiensis* in this study; (B–D) Mangrove forest of Shuidong bay, Dianbai County, Guangdong province, where the holotype and paratypes of *C. dianbaiensis* were collected.

3.2.3 Variability in shell characters

The shell morphology of *C. dianbaiensis* is phenoplastic as seen in other congeneric oysters. The left valve is usually deeply cupped, and the right valve is usually slightly concave. The body is medium size (approximately 5–10 cm in height, based on 20 individuals). The adductor muscle scars vary in color from dark purple to white in the right valve, but always show white in the left valve.

3.2.4 Anatomy

As described by Torigoe (1981), the anatomy of *C. dianbaiensis* is indistinguishable from that of other identified *Crassostrea* species.

3.2.5 Ecology

C. dianbaiensis occurs as wild individuals on mangroves in the intertidal zone and subtidal zone along the shoreline of Shuidong Gulf, Dianbai County, Guangdong province and Qinglan Harbor, Hainan province (Fig. 3A). In this study, all specimens were collected from a local tidal mangrove forest (Fig. 3B-D). In Dianbai, *C. dianbaiensis* is sympatrically distributed with four other species, *C. sikamea*, *C. angulata*, *C. ariakensis* and *C. hongkongensis*, and appears to have niche overlap with *C. sikamea* and *C. angulata*. In Qinglan Harbor, however, only two *Crassostrea* species were collected from the intertidal mangrove forest, namely *C. dianbaiensis* and *C. iredalei*.

3.2.6 Etymology

This is the first new species of Ostreidae recorded from Dianbai County, Guangdong province, China, and is thus named for the locality at which the oyster was originally found.

4 Discussion

We identified *C. dianbaiensis* as a distinct species based on the following evidence. First, mtDNA sequence-based phylogenetic analysis unambiguously confirmed its unique taxonomic status as a new member of the Indo-Pacific *Crassostrea*. Second, studies of the morphological characteristics of the shells of *C. dianbaiensis* and other Indo-Pacific *Crassostrea* species have shown that there are several unique features that distinguish the new species from other known species. In particular, those sympatric species include *C. angulata*, *C. sikamea* and *C. iredalei*. Third, we reviewed all *Crassostrea* species listed in WoRMS[1], WMSDB[2], Encyclopedia of Life (eoL) and in Huber (2010), with morphological descriptions, photographs and other information (e.g., habitat and distribution) available in these databases. We then carefully compared the characteristics of each species (a total of 35 species) with those of the new taxon, and it unambiguously showed that *C. dianbaiensis* differs in many aspects from each of these known *Crassostrea* species.

Based on the *cox1* phylogenetic tree, the unassigned oyster (*Crassostrea* sp. 1) found in Wenchang, Hainan province by Liu et al. (2011) should be recognized as *C. dianbaiensis* because these two taxa share almost identical *cox1* sequences.

[1] World Register of Marine Species.
[2] Worldwide Mollusc Species Data Base.

Phylogenetically, the Dianbai oyster is closely related with four other tropical oysters. These five tropical *Crassostrea* oysters (*C. belcheri*, *C. iredalei*, *C. gryphoides*, *C. madrasensis* and *C. dianbaiensis*) are coincidently all distributed in the mangrove ecosystem. Compared to the other four tropical oysters, the Dianbai oyster has the northernmost distribution boundary (Fig. 2A). The slipper-shaped oyster *C. iredalei* and the white scar oyster *C. belcheri* are widely spread and commercially farmed in Thailand, Malaysia and the Philippines (Bussarawit 2003), while only *C. iredalei* is sporadically distributed alongside the southernmost province in China, Hainan province (Xia 2008). Both *C. iredalei* and *C. dianbaiensis* have a low population density in the region (Xia 2008). For *C. dianbaiensis*, in particular, only 24 living individuals were collected from two of 15 locations during our field investigation (Xia 2008; Table 1). A possible explanation is that *C. dianbaiensis* is primarily distributed in the tropical areas and sporadically distributed in adjacent regions with the aid of ocean currents. Therefore, as the newest member of *Crassostrea* oysters, future studies are required to better understand more about its faunal characteristics, population structure and role in nearshore ecosystems.

In this study, two Indian oysters, *C. gryphoides* and *C. madrasensis* cannot be inferred as having closer relationships despite their sympatric distribution. However, the genetic relationship between *C. madrasensis* and *C. iredalei* is very close (1.5% *cox1* sequence divergence), even comparable with that (2.4% divergence) between *C. angulata* and *C. gigas*, which have recently been suggested as two subspecies of the Pacific oyster *C. gigas* (Wang et al. 2010). As *C. iredalei* is also primarily distributed in India's coast (Bussarawit 2003), it is possible that *C. madrasensis* was misidentified as *C. iredalei*, or these two species are now undergoing allopatric speciation by geographical separation. More studies and extensive sampling from different locations for *C. madrasensis* and *C. iredalei* may help to solve this puzzle.

With the help of mitochondrial DNA, we can effectively screen out the key morphological characteristics for species identification. For example, *C. ariakensis* from northern China is genetically distinct from *C. ariakensis* from southern China, and morphologically (to some extent), the southern *C. ariakensis* has purple adductor muscle scars, while northern *C. ariakensis* has white adductor muscle scars (Wang et al. 2004). The adductor muscle scar is the most conspicuous and important stress distribution area on the inner shell; its shape, position and color are peculiar to each species (Bussarawit 2003; Song et al. 2013). A typical example of using the adductor muscle scar as a key species identification characteristic is *C. belcheri*, which has a crescentic shaped, white adductor muscle scar for both valves. Both *C. iredalei* and *C. madrasensis* have a kidney-shaped, black or purple adductor muscle scar (Bussarawit 2003). Siddiqui and Ahmed (2002) reported that *C. madrasensis* and *C. gryphoides*, recognized in Pakistan, cannot be separated from each other on the basis of their external shell morphology, but can be separated by the inner shell with respect to the purpleness of the adductor muscle scar in *C. madrasensis* and the white color of both vales in *C. gryphoides*. Batista et al. (2008) also claimed that the differences in muscle scar pigmentation observed between *C. angulata* and *C. gigas* support the distinction of these two closely related taxa. In this study, the adductor muscle scars of *C. dianbaiensis* varied in color from dark purple to white in the right valve, but always showed white in the left valve.

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