Mar Biol
DOI 10.1007/s00227-015-2637-5

ORIGINAL PAPER

# Molecular phylogenetics reveals first record and invasion of *Saccostrea* species in the Caribbean

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Received: 29 August 2014 / Accepted: 17 February 2015


**Abstract** Taxonomic uncertainty often limits our ability to resolve biogeographic patterns and discern biological invasions. Within the bivalve mollusks, this uncertainty is particularly acute for oysters, as the high degree of phenotypic plasticity of their shells creates taxonomic confusion. The integration of molecular data with shell morphology can differentiate species, providing new insights into biogeography, invasions, and ecology of this functionally important group. As an initial step in resolving the identities and current geographic distributions of oyster species, sequence data from the mitochondrial cytochrome oxidase I gene were combined with morphological criteria to confirm the identities of ten oyster species of Ostreidae, Isognomonidae, and Pteriidae, focusing on the Pacific and Caribbean coasts of Panama, since tropical biota have received the least study. The results indicate that *Crassostrea virginica*, previously only reported from this region along the Yucatan Peninsula and coast of Venezuela, also occurs in the Caribbean waters of Panama. We also document the first record for a species of *Saccostrea*, a genus native to the Pacific, suggesting an invasion by an unknown non-native *Saccostrea* species that is now widespread along the Caribbean from the Panama Canal west to Bocas del Toro. Sequences of the internal transcribed spacer region (ITS1) of the ribosomal gene complex (rDNA) did not reveal any hybridization. Considering the high connectivity of shipping and boating in Panama, *Saccostrea* sp. may have been introduced to the Caribbean by either recreational or commercial vessels, but the timing and potential ecological effects of this invasion remain unknown.

Communicated by C. Riginos.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00227-015-2637-5) contains supplementary material, which is available to authorized users.

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## Introduction

Oysters are ecosystem engineers, playing many vital roles in coastal ecological processes, including nutrient cycling and benthic-pelagic coupling (Newell 2004), helping decrease eutrophication (Kemp et al. 2005), and creating habitat that serves as nurseries for commercially important fish and crab species (Coen et al. 2007). Additionally, oysters serve as hosts for a variety of protistan parasites, some of which can cause massive host mortalities and alter community structure and ecosystem dynamics (Villalba et al. 2004; Burreson and Ford 2004; Carnegie and Cochennec-Laureau 2004). Although oysters contribute greatly to

Published online: 10 March 2015

integral biotic processes in coastal waters, the geographic boundaries of many species are not well understood, and their taxonomy is often unclear. This is mainly due to their phenotypically plastic shells, which can be greatly influenced by environmental factors (Tack et al. 1992; Wilk and Bieler 2009), making species difficult to identify and circumscribe based on external shell characteristics alone.

To add to the confusion, oyster species often have widespread and overlapping geographic ranges, reflecting both natural and anthropogenic mechanisms for dispersal. Many bivalve larvae have extended planktonic stages, enabling them to disperse to distant locations (Scheltema 1986). Juvenile and adult oysters rafting on floating debris is another potential mechanism for the natural dispersal of some species, sometimes over vast distances (e.g., Ó Foighil et al. 1999; Donald et al. 2005). Bivalves can also be spread inadvertently via maritime traffic by attaching to the hulls of vessels (Gollasch 2002; Carlton 1992; Davidson et al. 2009), as larvae in ballast water (Gollasch et al. 2002; Briski et al. 2012), or associated with sea chests (Coutts et al. 2003; Coutts and Dodgshun 2007). Additionally, many bivalve species have been moved deliberately for aquaculture purposes (McKindsey et al. 2007) and through the aquarium trade (Padilla and Williams 2004; Weigle et al. 2005). All of these mechanisms aid in dispersing bivalve species and modifying their distributions, which, when combined with the inherent difficulties of morphological identification, can contribute to confusion in understanding the geographic range of species or recognizing new invaders.

Increasingly, molecular markers are being used as an independent data source for species identification, to detect cryptic or unrecognized species, and to clarify persistent taxonomic and biogeographic uncertainty. Molecular markers have been successfully used to differentiate members within the genus *Crassostrea* (e.g., Lam and Morton 2003; Reece et al. 2008; Cordes et al. 2008; Sekino and Yamashita 2013) and the genus *Saccostrea* (e.g., Lam and Morton 2006; Sekino and Yamashita 2013), and between other closely related oyster genera (e.g., Klinbunga et al. 2005; Liu et al. 2011), as well as aiding in resolving the distributions of other bivalve species. For example, sequence data confirmed the Trans-Atlantic geographic ranges of *C. rhizophorae* and *C. gasar* (Lapégue et al. 2002), as well as the identities and geographic ranges of multiple *Crassostrea* species along the Brazilian coast (de Melo et al. 2010). Furthermore, molecular markers have been used to confirm the introduction of non-indigenous bivalves through anthropogenic activities, such as the previously unrecognized introduction of the Pacific jingle shell, *Anomia peruviana*, to Caribbean waters (Schlöder et al. 2013) and detect potentially non-native species of *Crassostrea* in Brazilian waters (de Melo et al. 2010; Galvão et al. 2013).

As the first step in a study to resolve the broad-scale biogeography of oysters and their associated parasites across North and Central America, we used a combination of morphological criteria and sequences of the mitochondrial COI gene from multiple oyster species within the family Ostreidae and several families within the Pterioidea, focusing on commonly occurring species from multiple locations along the Pacific and Caribbean coasts of Panama. Our goals were to confirm the identities of the bivalve species collected and to determine their distributions within Panamanian waters.

## Materials and methods

### Specimen collection

We collected oysters from a variety of intertidal and subtidal habitats (primarily mangrove rhizophores, rocks, docks, pilings, etc.) from multiple locations on the Pacific and Caribbean coasts near the Panama Canal and at Bocas del Toro (Fig. 1). Maps showing the distribution of three of the identified bivalves and all sampling locations were generated using ArcGIS 10.2.2 for Desktop (Esri, Redlands, California). At the time of collection, physical data associated with the water were also collected (Electronic Supplementary Material (ESM) Table S1). We placed oysters in coolers on ice and, upon returning to the laboratory, kept the oysters at ~4 °C for no more than 72 h. Prior to the tissue sampling, we removed epibionts and mud from the shells, then measured and shucked the oysters. Oysters were tentatively identified in the field to the lowest possible taxonomic level (genus or species), using standard bibliographic references [e.g., Romashko (1992), Tucker Abbott and Morris (1995), Kaplan (1982) and Coan and Valentich-Scott (2012)] and later compared with synoptic collections deposited in the National Museum of Natural History. For molecular analyses, we sampled pieces of gill, mantle, and digestive gland and preserved them in 95 % ethanol. The majority of shells were thoroughly cleaned, dried, labeled with unique identification numbers, and retained as vouchers.

### DNA extraction, PCR amplification, and sequencing

Following an overnight digestion with proteinase K, we extracted genomic DNA from all three tissues sampled, which were pooled into a single extraction, using a Qiagen Biosprint Kit (Qiagen, Valencia, California) following the manufacturer's protocols for animal tissues. All extractions completed within the same day included a blank extraction, which served as a negative extraction control for PCR.

PCR amplification was carried out using primers jgLCO1490 (5′-TNTCNACNAAYCAYAARGAYATTGG-3′)

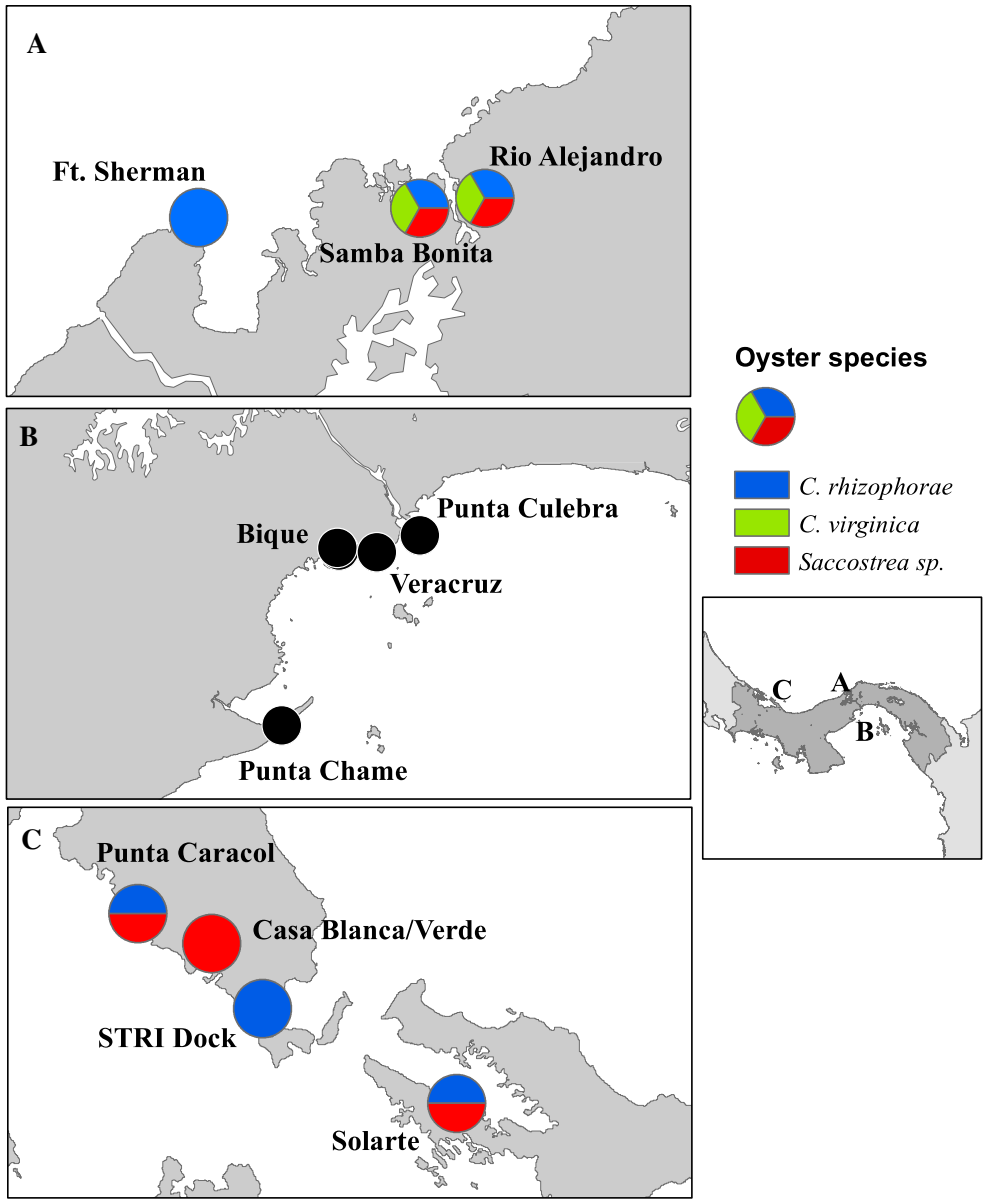


**Fig. 1** Map of sampling sites in Panama including **a** the Canal-Caribbean, **b** the Canal-Pacific, and **c** Bocas del Toro. The locations where *Saccostrea* sp., *C. virginica*, and *C. rhizophorae* were found are shown. The *black circles* indicate sampling locations where these three species were not detected. When either *Crassostrea* species or *Saccostrea* sp. was found, the *circles* are colored according to species. The percent *shading* corresponds to the number of species detected in each location and is not representative of the number of individuals or sequences per species

and jgHCO2198 (5′-TANACYTCNGGRTGNCCRAARAAYCA-3′) from Geller et al. (2013), which amplifies a >660 base pair (bp) fragment of the mitochondrial COI gene. PCR reagents consisted of 1X GeneAmp 10X PCR Gold Buffer (150 mM Tris–HCl, pH 8.0; 500 mM KCl; Applied Biosystems, Carlsbad, California), 2 mM $MgCl_2$, 0.2 mM of each nucleotide, 0.4 μM of each primer, 0.2 mg/mL of bovine serum albumin (BSA; New England Biolabs, Ipswich, Massachusetts), and 0.03 units/μL of AmpliTaq Gold with water to a final volume of 20 μL. Thermocycling was carried out using a Peltier Thermo Cycler DNA Engine Tetrad 2 (Bio-Rad, Hercules, California) with an initial denaturation of 94 °C for 10 min, 35 cycles of 94 °C for 1 min, 48–52 °C for 90 s, 72 °C for 1 min, and a final elongation of 72 °C for 5 min.

To ensure that the individuals identified as *Saccostrea* sp. with the COI sequences were not intergenic hybrids, we also amplified a subset of samples using ITS1-A (5′-GGTTTCTGTAGGTGAACCTGC-3′) and ITS1-B (5′-CTGCGTTCTTCATCGACCC-3′) (Hedgecock et al.

1999), which amplifies a >450 bp of the first internal transcribed spacer (ITS1) region of the ribosomal gene complex within the nuclear genome. PCR reagents consisted of 1X GeneAmp 10X PCR Gold Buffer (150 mM Tris–HCl, pH 8.0; 500 mM KCl; Applied Biosystems), 1.5 mM $MgCl_2$, 0.2 mM of each nucleotide, 0.5 μM of each primer, 0.2 mg/mL of bovine serum albumin (BSA; New England Biolabs), and 0.025units/μL of AmpliTaq Gold with water to a final volume of 20 μL. Thermocycling was carried out using a Peltier Thermo Cycler DNA Engine Tetrad 2 (Bio-Rad) with an initial denaturation of 94 °C for 10 min, 35 cycles of 94 °C for 30 s, 52–57 °C for 1 min, 72 °C for 45 s, and a final elongation of 72 °C for 5 min. For each assay, an aliquot of the PCR product (5μL) was electrophoresed on an agarose gel (2 % w/v) stained with GelRed (Phenix Research, Candler, North Carolina) and visualized under UV light.

We directly cycle sequenced all amplified fragments, in both the forward and reverse directions, using the Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Inc.) and sequenced the amplicons on an ABI 3130 Sequencer (Applied Biosystems, Inc.).

### Sequence analysis

Sequences were edited using Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, Michigan). All COI sequences were checked for open reading frames, and all duplicate sequences for each putative species were removed prior to the phylogeny constructions. Sequences were aligned in Geneious 7.1.5 (Biomatters, Ltd., San Francisco, California) with MUSCLE (Edgar 2004) or MAFFT (Katoh et al. 2002; Katoh and Toh 2008) using default parameters, with minor manual adjustments as necessary. The COI alignment for the Ostreidae dataset was 655 bp, the *Pinctada* dataset was 651 bp, and the *Isognomon* dataset was 609 bp; the ITS1 alignment for *Saccostrea* was 757 bp. The extended length of the ITS1 alignment is due to the presence of many large indels when all three genera are included. To determine whether these indels influenced the results, we used the Gblocks server (Castresana 2000) with both more stringent (not allowing many contiguous non-conserved positions) and less stringent (allowing smaller final blocks, gap positions within the final blocks, and less strict flanking positions) parameters to remove the less conservative portions of the alignment. The resulting alignments (more stringent = 184 bp, less stringent = 463 bp) were subjected to the same or similar analyses as the original alignment (see below). The resulting phylogenetic trees were highly congruent for all alignments, so only the results of the full alignment (757 bp) are shown. JModeltest 2.1.4 (Darriba et al. 2012) was used to determine the best substitution models (ESM Table S2) for both COI and ITS1 alignments based on Akaike information criterion (AIC) corrected values using the appropriate number of available substitution models for Bayesian and maximum likelihood (ML) analyses. Bayesian analyses for each dataset using the suggested substitution model were performed in Geneious 7.1.5 with MrBayes 2.0.6 (Ronquist and Huelsenbeck 2003) using the default parameters for the *Isognomon* and *Pinctada* datasets. For the Ostreidae dataset, the parameters were changed to 2,200,000 generations, with a 200,000 tree burn-in, and sampling every 400 trees. Maximum likelihood analyses for each dataset with the suggested substitution model were also performed in Geneious 7.1.5 using PhyML (Guindon et al. 2010) with the BEST topology search option and 1000 bootstrap replicates.

Distance analyses were conducted using *MEGA* v6 (Tamura et al. 2013) for the COI and ITS1 alignments separately. For the ITS1 calculations, a separate alignment containing only the ITS1 sequences for the *Saccostrea* species was generated as described above. For both the within species (ITS1 only) and between species (COI, ITS1) calculations, the number of base pair differences per site was averaged over all sequence pairs (using 500 bootstrap replicates, maximum composite likelihood method, allowing for transitions and transversions, using uniform rates among sites, and removing all ambiguous positions for each sequence pair).

**Fig. 2** Phylogram for Ostreidae constructed using newly generated ▶ COI sequences (in *bold*) and GenBank data; the HKY+I+G substitution model for the Bayesian analysis, and TrN+I+G model for the maximum likelihood analysis, produced highly similar topologies. Sequences from this study are in *bold*. Posterior probabilities followed by bootstrap values are included at the nodes. If only a single value is present, it is the posterior probability. See text for additional details

## Results

We generated 174 COI sequences (GenBank accession numbers KP455010–KP455072), including 63 different haplotypes representing seven species within the Ostreidae, two species of *Isognomon* (Isognomonidae), and one species of *Pinctada* (Pteriidae) (Figs. 2, 3, 4; Table 1). There were 47 haplotypes that were unique to a single location (Table 1). Bayesian and maximum likelihood analyses of each dataset produced highly similar topologies, particularly for the nodes with high support. No species were found to occur on both the Caribbean and Pacific coasts of Panama.

On the Pacific coast, we recovered one species each belonging to the genera *Striostrea* (morphologically identified as *S. prismatica*), *Saccostrea* (morphologically identified as *S. palmula*), and *Crassostrea* (morphologically


GBMLB0088 Isognomon legumen
KF284062 Pinctada radiata
Striostrea prismatica
Striostrea
JF915500 Saccostrea sp.
AY038076 Saccostrea cucullata
AB748839 Saccostrea mordax
AB748838 Saccostrea mordax
Saccostrea sp.
Saccostrea
AB748836 Saccostrea cucullata
AB748835 Saccostrea cucullata
AB721961 Saccostrea cucullata
EU816089 Saccostrea cucullata
EU007483 Saccostrea glomerata
AB748829 Saccostrea cucullata
AB748828 Saccostrea cucullata
AB076910 Saccostrea kegaki
Saccostrea palmula
FJ262984 Saccostrea cucullata
AY038077 Crassostrea belcheri
FJ428750 Crassostrea madrasensis
AY038078 Crassostrea iredalei
AY160753 Crassostrea ariakensis
AY160752 Crassostrea ariakensis
AF300616 Crassostrea nippona
EU007459 Crassostrea hongkongensis
FJ717608 Crassostrea gigas
AF280608 Crassostrea gigas
AB641325 Crassostrea sikamea
AB641326 Crassostrea sikamea
AB641324 Crassostrea sikamea
Crassostrea rhizophorae
FJ717614 Crassostrea rhizophorae
FJ717612 Crassostrea rhizophorae
FJ717613 Crassostrea rhizophorae
FJ717639 Crassostrea rhizophorae
HM003496 Crassostrea rhizophorae
Crassostrea virginica
EU007485 Crassostrea virginica
JX468937 Crassostrea virginica
FJ973634 Crassostrea virginica
FJ973635 Crassostrea virginica
FJ973662 Crassostrea virginica
FJ973670 Crassostrea virginica
AY376633 Crassostrea virginica
FJ717611 Crassostrea gasar
FJ717642 Crassostrea brasiliana
FJ717640 Crassostrea brasiliana
FJ717641 Crassostrea brasiliana
Crassostrea columbiensis
Crassostrea
AB898289 Ostrea futamiensis
AB898290 Ostrea futamiensis
NC015231 Ostrea denselamellosa
JF301920 Ostrea chilensis
JF301917 Ostrea chilensis
AF120651 Ostrea edulis
DQ078639 Ostrea angasi
DQ078640 Ostrea angasi
DQ078638 Ostrea angasi
DQ464125 Ostrea (=Ostreola) conchapila
NC022688 Ostrea lurida
DQ226521 Ostrea puelchana
DQ226532 Ostrea (=Cryptostrea) permollis
DQ226531 Ostrea (=Cryptostrea) permollis
DQ313181 Ostrea (=Ostreola) stentina
JQ027292 Ostrea sp.
JQ027291 Ostrea sp.
DQ226533 Ostrea stentina (=aupouria)
AY376607 Ostrea stentina (=equestris)
DQ640082 Ostrea stentina (=equestris)
Dendostrea frons
KC683511 Dendostrea crenulifera
JF915514 Ostrea sp.
JF915513 Ostrea sp.
AB076908 Lopha cristagalli
AB898294 Ostrea circumpicta
AB084109 Dendostrea frons
Ostrea/Dendostrea/Lopha
0.5

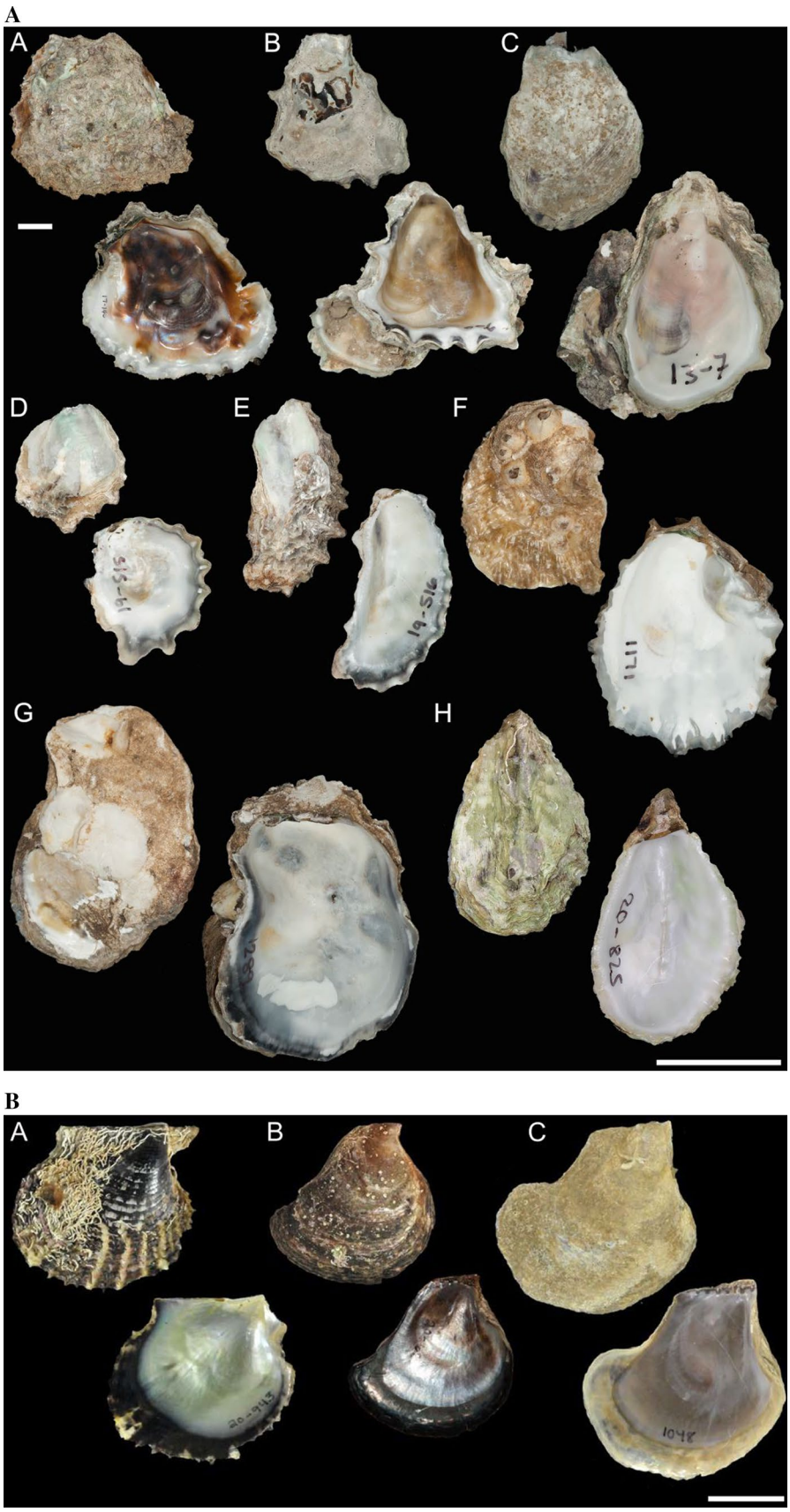


**Fig. 3** Representative vouchers for **a** all seven species of Ostreidae and **b** the three species of Pterioidea that were collected and sequenced from Panamanian waters. The species shown in 3A are as follows: A. *Striostrea prismatica*, B. *Saccostrea palmula*, C. *Crassostrea columbiensis,* D. *Saccostrea* sp., E. *Saccostrea* sp., F. *Crassostrea virginica,* G. *Crassostrea rhizophorae,* H. *Dendostrea frons*. The species shown in 3B are as follows: A. *Pinctada imbricata*, B. *Isognomon alatus*, C. *Isognomon* sp. *Scale bars* 2 cm

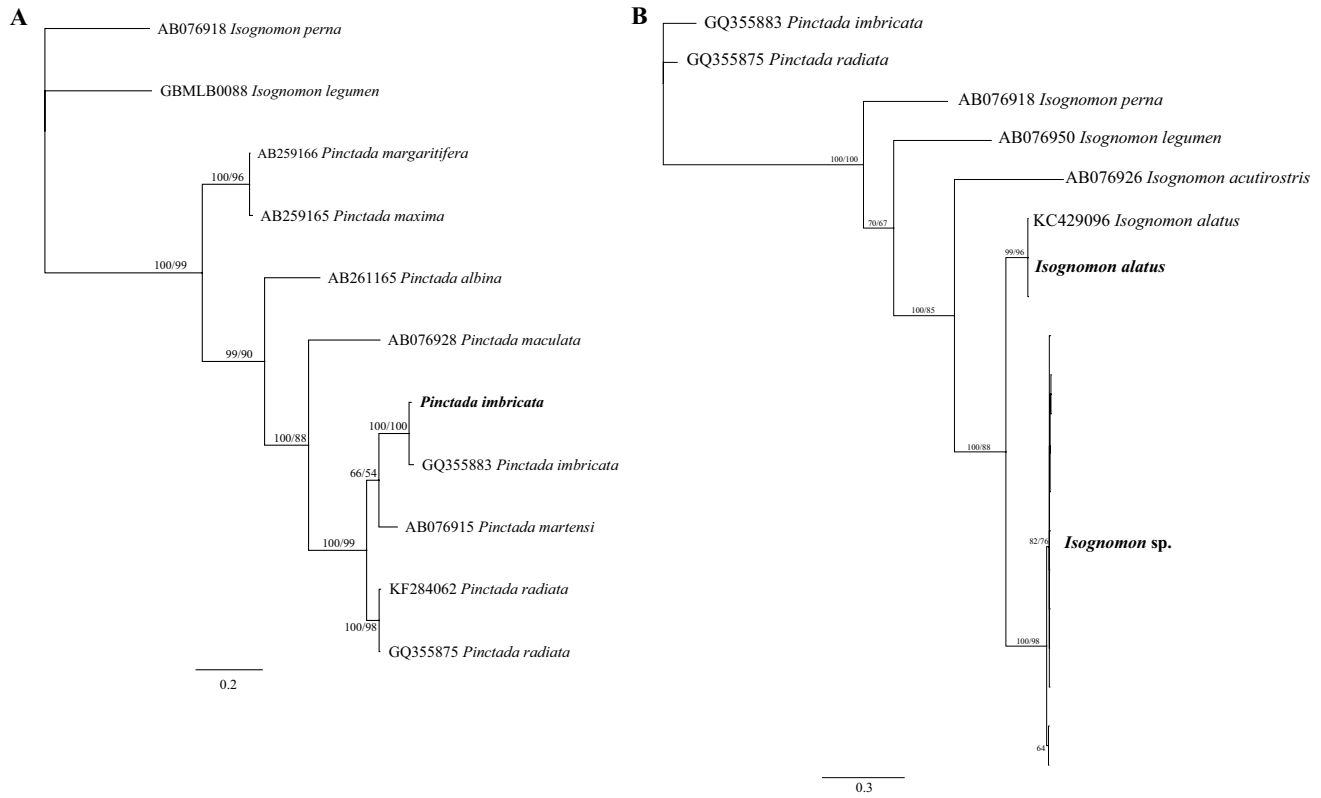


**Fig. 4** Phylograms for *Pinctada* (**a**) and *Isognomon* spp. (**b**) were constructed using newly generated COI sequences (in *bold*) and GenBank data. For *Pinctada*, the HKY+I+G substitution model for the Bayesian analysis, and the TIM3+I+G model for the maximum likelihood analysis, produced identical topologies. For *Isognomon*, the HKY+I+G substitution model for the Bayesian analysis and the TrN+I+G model for the maximum likelihood analysis produced identical topologies. Posterior probabilities followed by bootstrap values are included at the nodes. See text for additional details

identified as *C. columbiensis*); unfortunately, there are no previously published COI sequences of these species for comparison (Fig. 2). On the Caribbean coast, we recovered two species of *Crassostrea*, one species of *Dendostrea* (morphologically identified as *D. frons*), and one species of *Saccostrea*. Although all *Crassostrea* individuals were tentatively identified in the field as *C. rhizophorae*, subsequent morphological and molecular (COI) analyses indicated that both *C. virginica* and *C. rhizophorae* are present on the Caribbean coast, and that the two occur in sympatry (Figs. 1, 2). Average genetic distance values for the COI sequences between all species of *Crassostrea* ranged from 0.01–0.45 (±0.0 − 0.19 SE), with the average genetic distance between the *C. rhizophorae* sequences from this study and previously published sequences being 0.01 (±0.00 SE). Additionally, the average genetic distance between *C. virginica* sequences from this study and previously published sequences was 0.03 (±0.01 SE).

COI sequences of individuals identified as *Saccostrea* from the Caribbean coast were found to be genetically distinct from *S. palmula* from the Pacific coast and clustered with sequences obtained from GenBank identified as *S. cucullata* from Japan and the South China Sea (Fig. 2), from published (Sekino and Yamashita 2013) and unpublished sources. However, all *S. cucullata* COI sequences obtained from GenBank do not form a monophyletic clade (see "Discussion"). The average genetic distance values for the COI sequences between all species of *Saccostrea* ranged from 0.004 to 0.23 (±0.0012−0.08 SE), with the average genetic distance between the *Saccostrea* sp. from the Caribbean and the *S. cucullata* sequences in the same clade being 0.004 (±0.0012 SE).

Based on morphological criteria, we identified one oyster collected from Bocas del Toro as *D. frons*, although the sequence for this individual does not cluster with the only COI sequence obtained from GenBank from an unpublished source (Fig. 2). Average genetic distance values for the COI sequences between all species of *Dendostrea* ranged from 0.17 to 0.23 (±0.06 − 0.08 SE). The average genetic distance between the *D. frons* sequences from this study and previously published sequences was 0.17 (±0.08 SE).

Also on the Caribbean side of Panama, the morphological and molecular results confirmed the identification

**Table 1** Oyster species collected from each location, with the total number of cytochrome oxidase I (COI) sequences generated, total number of haplotypes from each species in each location, and the number of unique haplotypes from each location

| Region | Location | Species | Total COI sequences | Total COI haplotypes | Unique COI haplotypes | Total ITS1 sequences | Total ITS1 haplotypes | Unique ITS1 haplotypes | GenBank Accession Numbers |
|---|---|---|---|---|---|---|---|---|---|
| Canal-Pacific | Punta Culebra | *Saccrostrea palmula* | 6 | 4 | 2 | | | | KP455 012, KP455013, KP455057, KP455 058 |
| | Bique Mangroves | *Crassostrea columbiensis* | 9 | 3 | 2 | | | | KP455 017, KP455054, KP455055 |
| | | *Saccrostrea palmula* | 1 | 1 | 1 | | | | KP455056 |
| | Bique Intertidal | *Striostrea prismatica* | 9 | 2 | 1 | | | | KP455 010, KP455053 |
| | | *Saccrostrea palmula* | 6 | 5 | 3 | | | | KP455 012, KP455013, KP455047-KP455049 |
| | Veracruz | *Striostrea prismatica* | 5 | 3 | 2 | | | | KP455 010, KP455045, KP455046 |
| | Punta Chame | *Crassostrea columbiensis* | 5 | 3 | 2 | | | | KP455 017, KP455051, KP455052 |
| Bocas del Toro | Punta Caracol | *Crassostrea rhizophorae* | 5 | 1 | 0 | | | | KP455016 |
| | | *Saccostrea* sp. | 10 | 5 | 4 | 5 | 5 | 4 | KP455 011, KP455 041-KP455044; KP455074, KP455087-KP455090 |
| | | *Isognomon* sp. | 6 | 6 | 1 | | | | KP455061- KP455063, KP455065, KP455066, KP455072 |
| | Solarte | *Crassostrea rhizophorae* | 6 | 1 | 0 | | | | KP455016 |
| | | *Saccostrea* sp. | 10 | 6 | 5 | 7 | 7 | 5 | KP455 011, KP455036-KP455 040; KP455073, KP455076, KP455082-KP455086 |
| | | *Isognomon* sp. | 5 | 4 | 2 | | | | KP455065, KP455066, KP455070, KP455071 |
| | STRI Dock | *Crassostrea rhizophorae* | 5 | 1 | 0 | | | | KP455016 |
| | | *Dendostrea frons* | 7 | 2 | 1 | | | | KP455 014, KP455059 |
| | | *Pinctada imbricata* | 7 | 1 | 0 | | | | KP455060 |
| | | *Isognomon alatus* | 4 | 2 | 1 | | | | KP455064, KP455069 |
| | Casa Blanca/ Verde | *Saccostrea* sp. | 11 | 6 | 5 | 6 | 4 | 1 | KP455011, KP455031-KP455035; KP455073-KP455075, KP455081 |
| Canal-Caribbean | Fort Sherman | *Crassostrea rhizophorae* | 6 | 2 | 1 | | | | KP455016, KP455050 |
| | | *Isognomon* sp. | 5 | 4 | 0 | | | | KP455063, KP455065, KP455066, KP455067 |
| | Rio Alejandro | *Crassostrea rhizophorae* | 5 | 3 | 2 | | | | KP455 016, KP455026, KP455028 |
| | | *Crassostrea virginica* | 4 | 2 | 1 | | | | KP455 015, KP455029 |
| | | *Saccostrea* sp. | 11 | 3 | 2 | 5 | 4 | 2 | KP455011, KP455 027, KP455030; KP455 075,P455076, KP455079, KP455080 |
| | | *Isognomon* sp. | 5 | 4 | 1 | | | | KP455062, KP455063, KP455067, KP455068 |
| | Samba Bonita | *Crassostrea rhizophorae* | 5 | 2 | 1 | | | | KP455 016, KP455025 |
| | | *Crassostrea virginica* | 4 | 3 | 2 | | | | KP455015, KP455023, KP455024 |
| | | *Saccostrea* sp. | 7 | 6 | 5 | 6 | 3 | 2 | KP455 011, KP455 018-KP455022; KP455076, KP455077, KP455078 |
| | | *Isognomon* sp. | 5 | 3 | 0 | | | | KP455061, KP455063, KP455065 |
| | | Totals | 174 | | 47 | 29 | | 14 | |

The total number of internal transcribed spacer region (ITS1) sequences, including total and unique haplotypes generated for each location for *Saccostrea* sp., is also listed

of *Pinctada imbricata* from Bocas del Toro (Fig. 4a). The genetic distance between the *P. imbricata* sequences from this study and previously published sequences was 0.02 (±0.01 SE). In addition, our results confirmed the presence of *Isognomon alatus* (Fig. 4b) from the STRI Dock. The average genetic distance between the *I. alatus* sequences from this study and previously published sequences was 0.00 (±0.00 SE). We also found another species, *Isognomon* sp. (Wilk, unpubl. data), which was widespread through Panamanian waters (Fig. 4b; Table 1).

We generated 29 ITS1 sequences (GenBank accession numbers KP455073–KP455090) from the Caribbean *Saccostrea* species (Table 1), including 18 haplotypes, 14 of which were unique to a given location, which formed a monophyletic clade with published sequences of *Saccostrea* species (ESM Fig. S1), indicating that these individuals are not intergenic hybrids. Based on the ITS1 sequences, the average genetic distance between all species of *Saccostrea* ranged from 0.01 to 0.03 (±0.00−0.01 SE), the average genetic distance between *Saccostrea* sp. in the Caribbean and other *Saccostrea* species ranged from 0.020 to 0.028 (±0.006−0.01 SE), and the average genetic distance between *Saccostrea* sp. in the Caribbean and other *Saccostrea cucullata* sequences was 0.028 (±0.01 SE).

## Discussion

### First record of the genus *Saccostrea* in the Caribbean

We discovered a non-indigenous species of *Saccostrea*, apparently a member of the *S.* "*cucullata*" species complex that is native to the Indo-Pacific, is now widespread along the Caribbean coast of Panama. This is the first published record of a *Saccostrea* species occurring in the Caribbean Sea, with the geographic range and abundance indicating that it is established in the basin. The resulting COI sequences are virtually identical to sequences from *S. cucullata* collected in Japan and the South China Sea (Fig. 2), indicating a potential source for this invasion. *Saccostrea cucullata* was described from India, and currently is understood to be indigenous to rocky shores throughout the Indo-Pacific (Lam and Morton 2006) and along the south and east coasts of Africa (Haupt et al. 2010). However, COI and 16S sequences have demonstrated that *S. "cucullata"* represents a cryptic species complex comprising as many as seven species, often with broadly overlapping geographic distributions (Lam and Morton 2006; Sekino and Yamashita 2013). To further complicate the issue, *S. "cucullata"* is believed to have been introduced to a number of locations where it has become established, including Hawaii (Coles et al. 1999), the Mediterranean Sea, and the Suez Canal (Galil and Zenetos 2002). While our morphological and molecular data clearly demonstrate that the species we collected is a member of *Saccostrea*, a genus not indigenous to the Caribbean, assigning these individuals to the correct taxonomical species requires a comprehensive systematic revision of the *S. "cucullata"* complex and is beyond the scope of this analysis.

The uncertainty regarding taxonomic identity notwithstanding, we know that the *Saccostrea* species is not only present, but widespread in Panamanian waters, occurring at locations near the Caribbean entrance to the Panama Canal and at Bocas del Toro, >250 km apart (Fig. 1). When present, individuals were abundant, and typically found attached to mangrove rhizophores in close association with *C. rhizophorae*. We cannot definitively ascertain how and when *Saccostrea* was introduced into the Caribbean, especially since this species is not commercially harvested and was unlikely to be the result of an intentional introduction (for which there are sometimes records). However, Panama serves as a hub for international shipping and movement of recreational vessels (Ruiz et al. 2009), making it likely that this species was introduced via shipping or boating activity. Similarly, the Pacific jingle shell, *A. peruviana* (Schlöder et al. 2013) and the mud crab, *Rhithropanopeus harrisii* (Roche and Torchin 2007), were potentially introduced through maritime traffic in Panama.

### Expanded geographic range of *Crassostrea virginica*

Previous reports indicate the geographic range of *C. virginica* extends from the Gulf of St. Lawrence in Canada, south to the Gulf of Mexico, and also includes Venezuela (Stanley and Sellers 1986). This study expands the known geographic range for this species to include the Caribbean waters of Panama, specifically the coastal waters near the Panama Canal (Fig. 1).

Genetic markers were previously used to demonstrate that *C. virginica* and *C. rhizophorae* are sister taxa (Lazoski et al. 2011). These two morphologically similar species were thought to prefer different habitat types, with *C. virginica* occurring primarily in intertidal and subtidal depths attached to hard substrates (Stanley and Sellers 1986) and *C. rhizophorae* attached to the roots of mangrove trees from the Caribbean Sea south to Brazil (Carriker and Gaffney 1996). Indeed, Littlewood and Donovan (1988) attempted to differentiate between the two species based on habitat preference. In contrast, our data indicate that *C. virginica* and *C. rhizophorae* can and do co-occur in the same habitat, as both species were found growing on mangrove rhizophores. However, we only recovered *C. virginica* from two neighboring locations (Fig. 1), where it was found to occur in a much lower abundance compared to *C. rhizophorae*. Additional studies are needed to further assess the abundance and distribution of *C. virginica* in this

area, and to further explore its habitat preferences, especially in sympatry with *C. rhizophorae*.

Insights from the molecular phylogenies

As in previous studies, this work demonstrates the power of combining molecular with morphological data to confirm the identities of species that are difficult to delineate with morphological characters alone. Although COI sequences alone cannot be expected to robustly resolve deeper nodes within and among genera and families, our results are consistent with those of Tëmkin (2010), who also found high support, for the *Pinctada imbricata/fucata/radiata* species complex. However, the COI results for the *Isognomon* species in this study appear quite different from the results of Tëmkin (2010), which either reflects the limits of the resolving power of this marker or issues associated with inadequate taxon sampling (in this case, due to a lack of available COI sequences for *Isognomon* species). Our results are consistent with those of Wilk (unpubl. data), who has evidence of two sympatric species of *Isognomon* in peninsular Florida, *I. alatus,* and *Isognomon* sp. Additionally, the sequences we generated for *D. frons* did not form a monophyletic clade with the *D. frons* sequence obtained from GenBank, indicating this species may comprise a cryptic species complex; however, without more sequences or verification of the identity of the sequenced specimen cited in GenBank, this remains speculative.

Our results are also consistent with those of Polson et al. (2009), based on analysis of COIII and 16S mitochondrial sequences, and Shilts et al. (2007), based on 16S sequences, both of which supported the non-monophyly of *Ostrea*, *Dendostrea*, and *Lopha,* indicating that all three genera are in urgent need of comprehensive systematic revision.

## Conclusions

This study confirmed the presence of a non-native oyster that is established in the Caribbean and provides the first report of *C. virginica* in Panamanian waters. These results emphasize the need for major systematic revisions within the Ostreidae, namely resolving the species-level relationships within the *Saccostrea* "*cucullata*" complex as well as a re-examination of the monophyly and affinities of *Ostrea, Dendostrea,* and *Lopha*. Given the functional importance of oysters as ecosystem engineers and their vital roles in many coastal ecological processes (Newell 2004; Kemp et al. 2005; Coen et al. 2007), including detection of parasites and other problematic microbes (Ford et al. 2009), identifying the biogeographic distributions for these species is crucial to understanding and managing coastal ecosystems. Finally, this study highlights both the utility and opportunity of combining morphological and molecular tools to resolve identification and biogeography for oysters and other challenging taxonomic groups.

**Acknowledgments** We thank Gabriel Jacome and Plinio Gondola at the Smithsonian Bocas del Toro Marine Station for assistance and discussions on local bivalve species. Dr. Carmen Schlöder assisted with field collections and obtaining permits. We thank Dr. Ilya Tëmkin (NOVA) for assistance with identifying members of the Isognomonidae and John Wilk (UIC) for sharing his data and confirming the identity of the second species of *Isognomon*. Dr. Mark Minton assisted with the creation of maps in ArcGIS. Nancy Rotzel McInerney provided lab logistical support at CCEG. A Smithsonian Institution Grand Challenges Grant funded this project. A Smithsonian Institution MarineGEO Postdoctoral Fellowship funded KM Pagenkopp Lohan. This is contribution number 2 from the Smithsonian's Tennenbaum Marine Observatories Network.

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