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Morphological and genetic species diversity in ostracods (Crustacea: Oligostraca) from Caribbean reefs

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Abstract The taxonomy and ecology of ostracods in the Caribbean remain incomplete, even though they are among the most successful and ubiquitous microcrustaceans of marine ecosystems. In an effort to enhance our knowledge of the biodiversity, abundance, and distribution of benthic ostracods, several sediment samples were collected from mesophotic coral ecosystems (MCEs) of Puerto Rico and U.S. Virgin Islands at different depths (30–102 m) using technical diving. The highest densities of ostracods were found in the deepest samples (≥61 m), and these were the most abundant and diverse assemblages. All ostracods collected belong to the subclasses Myodocopa Sars and Podocopa Sars. Myodocopa was represented by the families Cypridinidae Baird, Polycopidae Sars, Sarsiellidae Brady & Norman, Rutidermatidae Brady & Norman, Cylindroleberididae Müller, and Philomedidae Müller. On the other hand, Podocopa was represented by the following families: Bairdiidae Sars, Pontocyprididae Müller, Candonidae Kaufmann (subfamily Paracypridinae Sars), Macrocyprididae Müller, Loxoconchidae Sars, Xestoleberididae Sars, Cytherellidae Sars and Cytheromatidae Elofson. The subclass Podocopa showed the highest number of individuals and species. There was a ~100 % correspondence between morphologically identified species and genetically defined species through a short region of the nuclear large subunit ribosomal DNA, which was shown to be appropriate for species recognition and discrimination in ostracods. Using a morphological (shell shape and ornamentation) and molecular barcoding approach (28S rDNA), we provide the first report of the biodiversity of ostracods in the mesophotic coral ecosystems of northeastern Caribbean.

Keywords Ostracoda · Mesophotic coral ecosystems · Morphology · Molecular Barcoding · Caribbean · Puerto Rico · U.S. Virgin Islands

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Introduction

Mesophotic coral ecosystems are light-dependent benthic communities starting at 30–40 m and extending to over 150 m depth; they are usually found in the upper insular and continental slopes of the Caribbean region (Locker et al. 2010; Sherman et al. 2010, 2013; Appeldoorn et al. 2015). The structural habitat associated with the mesophotic zone includes coral, sponge, and algal communities (Hinderstein et al. 2010). The substrata that support mesophotic coral communities consist of limestone structures and rhodolith banks providing a suitable hard substrate when agglutinated into solid banks by red coralline algae (Fricke and Meischner 1985; Littler et al. 1991; García-Sais et al. 2008; Rivero-Calle et al. 2008). Advances in technical scientific diving and instrumentation such as mixed gas diving with rebreathers provide access to work safely on depths unattainable with traditional SCUBA (Sherman et al. 2009, 2013; Jessup 2014; Appeldoorn et al. 2015) and sample specific substrata minimizing damage of the habitat.

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Interest in studying these ecosystems has increased greatly during recent years because they may play key roles as refugia for corals, fish, and many other species during times of environmental stress and a possible source of larvae that could replenish shallow water reefs (Riegl and Piller 2003; Lesser et al. 2009; Bongaerts et al. 2010; Kahng et al. 2014). We are particularly interested in the crustacean fauna, and several publications have been devoted to the crustacean biodiversity of mesophotic reefs (e.g., Petrescu et al. 2012, 2013; Corgosinho and Schizas 2013; Senna et al. 2014; Schizas et al. 2015). An abundant component of the mesophotic meiofauna are benthic Ostracoda, which are also well known for their remarkable fossil record (Benson and Coleman 1963; Ruppert et al. 2004; Cohen et al. 2007; Oakley et al. 2012; Rodriguez-Lazaro and Ruiz-Muñoz 2012), and usefulness as indicators of paleoenvironments (Benson and Coleman 1963; Alvarez et al. 2000; Martin-Rubio et al. 2006; Rodriguez-Lazaro and Ruiz-Muñoz 2012). They are highly diverse with around 25,000 extant species (Cohen et al. 2007). Ostracods can be found worldwide in almost any fresh and marine water body at different depths (Kornicker 1979; Machain 1996; Ruppert et al. 2004; Cohen et al. 2007). Most previous studies related to the biodiversity, ecology, zoogeography, and taxonomic classification of myodocopid ostracods have been focused in the Gulf of Mexico (Tressler 1954; Kornicker 1958, 1981, 1983, 1984a, b, 1986a, b; Machain 1996; Harrison-Nelson and Kornicker 2007). In the Caribbean Sea, studies on ostracods before 1965 were summarized in Baker and Hulings (1966); since then, ostracod fauna has been studied in the Bahamas, Cuba, U.S. Virgin Islands, Belize, Venezuela, Panama, Jamaica, Bermuda, and Dominican Republic. Podocopid ostracods have been studied by Maddocks (1969, 1990); she compiled a complete revision of recent Bairdiidae Sars, 1865 (Podocopa) and a comprehensive study of living and fossil Macrocyprididae Müller, 1912 (Podocopa) including collecting stations and geographic distributions of species in the western Atlantic, Caribbean, Gulf of Mexico, and many other marine regions. Previous works have been conducted in the Gulf of Mexico and Caribbean area (Benson and Coleman 1963; Maddocks 1969; McKenzie 1971; Maddocks 1974; Van den Bold 1974, 1977; Palacios-Fest and Gío Argáez 1979; Palacios-Fest et al. 1983; Van den Bold 1988; Maddocks 1990; Maddocks and Iliffe 1993; Maddocks et al. 2007).

In Puerto Rico (PR), the first studies on ostracods were focused on Podocopa Sars, 1866. Brady (1880) reported seven species of ostracods of the subclass Podocoda off Culebra Island, east Puerto Rico. Eighty-six years later, a study on the distribution of Podocopa around Puerto Rico was reported by Baker and Hulings (1966). Kornicker (1981, 1984b) studied the distribution and morphology of the subclass Myodocopa Sars, 1866 within the suborder Myodocopina Sars, 1866 and two species were collected from Puerto Rico. In 1990, the species *Macrosarisa texana* Maddocks, 1990 (family Macrocyprididae) collected off San Juan, PR was reported by Maddocks (1990). Two new species, *Diasterope puertoricensis* Morales-Núñez & Kornicker, 2007 (family Cylindroleberididae Müller, 1906) and *Euphilomedes chupacabra* Lum et al., 2008 (family Philomedidae Müller, 1906) were recently described from Culebra Island, PR (Morales-Núñez and Kornicker 2007) and La Parguera, PR (Lum et al. 2008).

The large number of ostracod specimens in our samples was the motivation to include DNA barcoding concomitantly with morphology to improve our biodiversity estimates of the mesophotic ostracod fauna. DNA barcodes are short DNA sequences retrieved from a specific genomic region (e.g., a fragment of the mitochondrial *COI* gene) of the taxon under study, useful for species recognition and discrimination (Hebert et al. 2003, 2004; Hajibabaei et al. 2006, 2007; Bucklin et al. 2011). In many cases (e.g., Nagy et al. 2012; Vasconcelos et al. 2016) high levels of cryptic diversity have been revealed; however, a critical shortcoming using small fragments of DNA to estimate the number of species was identified (Hickerson et al. 2006; Rubinoff et al. 2006; Dasmahapatra et al. 2010). Despite the drawbacks, DNA barcoding has become an important tool to biodiversity studies, especially if it is integrated in a taxonomic framework, because it provides a complementary and independent data set to morphology for the understanding of evolutionary and genetic relationships within and among species. Previous studies have shown the usefulness of the *COI* gene; Karanovic (2015) applied the barcoding approach using a fragment of the mtCOI to reconstruct the phylogeny of *Physocypria biwaensis* Okubo, 1990, an endemic ostracod from lake Biwa, Japan revealing cryptic speciation. Moreover, Nigro et al. (2016) used the same marker to analyze specimens of marine planktonic ostracods and showed the value of the *COI* barcode region for species discrimination, recognition, and identification. A study by Karanovic et al. (2015) provided phylogenetic analyses based on fragments of the nuclear 18S rDNA and 28S rDNA genes to support the position of the ostracod subfamily Pseudophilomedinae Kornicker, 1967 within the superfamily Sarsielloidea Brady & Norman, 1896. In our study we used a fragment of the nuclear 28S rDNA as barcode for species identification, which was used to ensure high rates of PCR amplification across the ostracods already in the past (Oakley and Cunningham 2002; Syme and Oakley 2012; Karanovic et al. 2015; Hiruta et al. 2016). We hypothesized that all morphologically different ostracod specimens, assigned to different species are also different at the molecular level, therefore, testing the concordance between morphological vs. genetic species concepts. Our study highlights the high biodiversity of benthic ostracod fauna encountered in the Caribbean mesophotic reefs, in an effort to promote awareness of this ecosystem in coastal conservation programs.

Materials and methods

Biological sampling design and processing

The samples were collected in a non-standardized way during the last 10 years, and the majority were obtained during three NOAA-funded research cruises with commercial diving vessels supporting mixed gas diving with rebreathers during 2010–2012 (Sherman et al. 2013). In these cruises, technical divers collected substrata from fragments of corals, sponges, and rhodoliths randomly from MCEs of west Puerto Rico [Bajo de Sico (BS), Tourmaline (TM) and Abrir la Sierra (ALS)], southwest Puerto Rico (El Hoyo, Precipicio, Black Wall, Hole-in-Wall, Weinberg and Barranco), Ponce Ledge, and the adjacent islands of Mona, Vieques, including the U.S. Virgin Islands of St. John and St. Croix (Fig. 1, Online Resource 1). Other sediment samples were collected from MCEs around the continental shelf off La Parguera, PR during 2007–2008 as part of the DeepCres program (Fig. 1, Online Resource 1). The mesophotic collection included samples from depths between 30 and 102 m.

In addition to the samples from MCEs, we included samples from randomly collected sediments from shallow water habitats from the Caribbean region and adjacent areas. Sediment samples or substrata samples were collected using traditional SCUBA from the following reef locations of Puerto Rico: La Parguera (Hole-in-Wall, Mata la Gata, Channels, Collado, San Cristóbal, and Margarita) and Caja de Muertos (Online Resource 2). For comparison we included other shallow water samples from St. John (U.S. Virgin Islands), Los Haitises (Dominican Republic), the Bahamas, Bermuda, Ft. Lauderdale (FL, USA), and Taboguilla Island (Pacific Panama). These shallow water collections included samples from depths between 3 and 25 m (Online Resource 2).

Each substrate sample was placed in a zip-locked bag with in situ sea water and labeled with the location, date, and depth. Benthic samples were processed immediately after collection either on the ship deck or at the Molecular Evolution of Marine Invertebrates Laboratory at Magueyes Island, UPRM. Samples were washed over a 1 mm and a 0.125 mm sieves and all the material retained on the smaller sieve was stored in 100 % ethanol. Ostracod specimens were hand sorted directly from the sediment or after a Ludox AM-30 colloidal silica resuspension and centrifugation step, used for mass-extraction of meiofauna and macrofauna (Nichols 1979) with the aid of a binocular microscope (Olympus SZH10) and stored in small plastic vials (1.5 mL) with 100 % ethanol in the refrigerator for future molecular work.

The ostracods were identified to superfamily or family level using a stereoscope (Olympus SZH10) and a compound microscope (Olympus BX51). Ostracods were characterized morphologically based mostly in the carapace morphology (shell shape and ornamentation) since the external anatomy provides important characters for identification. The surface of the carapace varies from smooth to highly ornamented; shape also varies greatly (circular, triangular, rectangular,

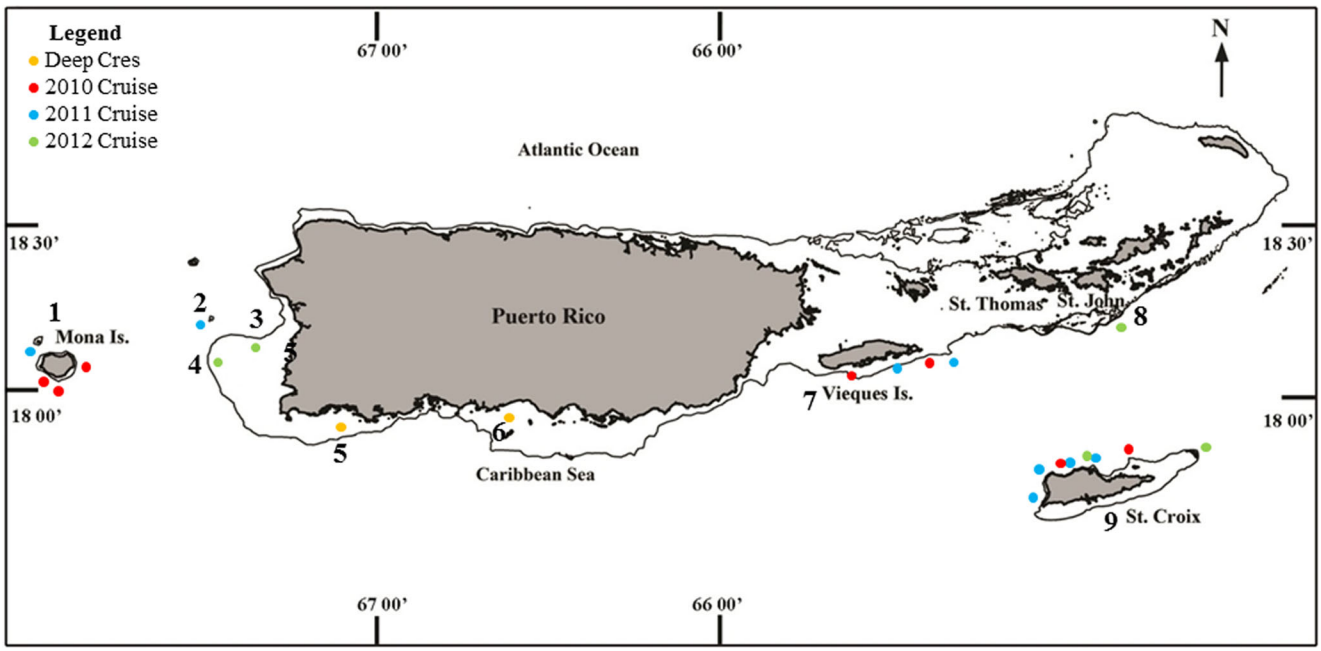

Fig. 1 Map of Puerto Rico (PR) and the U.S. Virgin Islands (USVI). The extended continental shelf (18–20 m depth) is noted by a thin *black line*. Numbers indicate the sampling locations of specimens collected from mesophotic coral ecosystems. 1 = Mona, PR, 2 = Bajo de Sico, PR, 3 = Tourmaline, PR, 4 = Abrir la Sierra, PR, 5 = La Parguera, PR, 6 = Ponce Ledge, PR, 7 = Vieques, PR, 8 = St. John, USVI, and 9 = St. Croix, USVI. Colored circles indicate the exact sampling locations (Online Resource 1). In *yellow*, the designated mesophotic locations of the DeepCres program; all other colors indicate samples collected during the 2010–2012 cruises (see Sherman et al. 2013). Map modified from Petrescu et al. (2016)

ovoid, reniform, etc.) (Kornicker 1979; Cohen 1982; Ruppert et al. 2004; Cohen et al. 2007). We also looked the first and second antennae in specimens that were difficult to identify only by their external anatomy. Specimens were examined according to the procedure used by Cohen et al. (2007). Ostracods were identified using the taxonomic studies of Kornicker (1981, 1983, 1984a, b, 1986a, b). The Key to the Ostracoda (Tabular keys to Myodocopa and Podocopa) by Cohen et al. (2007) was also used in the identification of specimens. Images of selected specimens were deposited in Morphbank (Online Resource 3).

Molecular procedures and analyses

The most abundant myodocopans and podocopans species were selected for molecular barcoding. We extracted DNA from ten myodocopan species [five collected from MCEs of Puerto Rico (species 22-2, 8, 11, 51 and 58) and five from shallow water habitats of Puerto Rico (species 22-3, 66, 67, 68 and 69)] belonging to four families (Cylindroleberididae, Philomedidae, Sarsiellidae Brady & Norman, 1896, and Rutidermatidae Brady & Norman, 1896; Table 1). For Podocopa, DNA was extracted from 22 species [18 collected from MCEs of Puerto Rico (species 2-1, 2-2, 2-3, 4, 5, 7, 14, 24-2, 26-1, 26-2, 40, 41, 44, 52, 54, 55, 59 and 60), one from MCEs of St. Croix USVI (sp. 26-3), and the remaining three from shallow water habitats of Panama (sp. 30), Dominican Republic (sp. 21), and Bermuda (sp. 18), respectively] (Table 1). The podocopan ostracods belonged to the superfamily Cytheroidea Baird, 1850 and eight families (Bairdiidae, Macrocyprididae, Pontocyprididae Müller, 1894, Xestoleberididae Sars, 1928, Cytheromatidae Elofson, 1939, Loxoconchidae Sars, 1925, Cytherellidae Sars, 1866, and Paradoxostomatidae Brady & Norman, 1889). Individual specimens of the same morphologically defined ostracod species were macerated in separate 1.5 mL centrifuge tube. DNA was extracted from the specimens preserved in 100 % ethanol using the DNeasy Blood & Tissue Kit (Qiagen Inc.) following the manufacturer's guidelines.

A region of the 28S rDNA was PCR-amplified using universal primer sets described in Hillis and Dixon (1991). The primers for 28S (v-x) were used because they have been shown to be variable at the species level in ostracods (Syme and Oakley 2012). The partial fragment D9-D11 represents the domain of the 28S (v-x) region (Machida and Knowlton 2012). PCR amplifications took place in 25 μL reactions containing 0.5 μL of DNA template, 0.5 μL (5 pmol) of each primer (28S v-x), 12.5 μL of 2x BioMix (Bioline Inc.) and 11 μL of molecular grade water. The PCR was run in a Bio-Rad thermal cycler machine; after the initial denaturation of 2 min at 94 °C, we programmed the thermal cycler to perform 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min and extension at 72 °C for 1 min and 30 s followed by a final extension at 72 °C for 6 min (Jarman et al. 2000). When specimens yielded identical sequences with the 28S v-x primers, we sequenced an additional region of 28S using the primers dd-ff (Hillis and Dixon 1991) with the same PCR conditions as outlined above. The additional 28S (dd-ff) region [partial fragment D3-D6 (Machida and Knowlton 2012)] was sequenced for 9 podocopan species collected from MCEs of Puerto Rico; the species belonged to the families Bairdiidae, Macrocyprididae, and Xestoleberididae (Table 2).

Successful PCR reactions were verified by loading 5 μL of the amplicon on a 1 % TBE agarose gel stained with ethidium bromide. Electrophoresis was carried out for 45 min at 73 V. Amplified bands were visualized under UV light and captured digitally. Successful amplicons were sequenced using an ABI 3130XL Genetic Analyzer.

DNA data analysis

DNA sequencing trace files were processed with Codon Code Aligner 5.0.1 for base calling, quality assessment, contig assembly, visualization, and manual editing. DNA sequences were aligned using MUSCLE (Edgar 2004) as implemented in MEGA 7 (Kumar et al. 2016) with the default parameters (gap opening penalty −400 and extend penalty 0); the resulting sequence alignments are available in Online Resource 4. Following quality control and end trimming, a final data set of 568 bp region of the 28S (v-x) and 651 bp region of the 28S (dd-ff) were used for phylogenetic and pairwise distance analyses. The resulting 28S sequences were imported into MEGA 7 to construct Maximum Likelihood (ML) trees. Within MEGA 7, the most appropriate model of DNA substitution for the 28S data sets were estimated and applied to the ML analysis [best substitution model for 28S (v-x) data set was K2+G+I and for 28S (dd-ff) K2+G]. Clade support was assessed with 1000 bootstrap replicates (Felsenstein 1985). Uncorrected pairwise distances between species were also estimated in MEGA 7. We deposited the DNA sequences in GenBank (Tables 1 and 2) and voucher specimens were placed in the Museum of Marine Invertebrates (MMI-UPRM) at Magueyes Island (Project Collection No. 10006).

Results

Biological

All the ostracods collected from Caribbean MCEs belonged to the subclasses Myodocopa and Podocopa. The Podocopa was the most representative subclass with 1536 individuals, whereas the subclass Myodocopa had 339 individuals. The Podocopa harbored a highest species number (total $n = 53$) than Myodocopa (total $n = 39$). The specimens were identified

Table 1 Species and GenBank accession numbers of 28S rDNA sequences (amplified with v-x primers) used in this study

Class	Subclass	Order	Species	GenBank No.
Ostracoda	Myodocopa	Myodocopida	Cylindroleberididae sp. 22–2	KP734265
Ostracoda	Myodocopa	Myodocopida	Cylindroleberididae sp. 22–3	KP734266
Ostracoda	Myodocopa	Myodocopida	Cylindroleberididae sp. 66	KP734267
Ostracoda	Myodocopa	Myodocopida	Cylindroleberididae sp. 67	KP410725
Ostracoda	Myodocopa	Myodocopida	Sarsiellidae sp. 51	KP734268
Ostracoda	Myodocopa	Myodocopida	Sarsiellidae sp. 8	KP410724
Ostracoda	Myodocopa	Myodocopida	Rutidermatidae sp. 11	KP734269
Ostracoda	Myodocopa	Myodocopida	Rutidermatidae sp. 68	KP734270
Ostracoda	Myodocopa	Myodocopida	Philomedidae sp. 58	KP734271
Ostracoda	Podocopa	Myodocopida	Philomedidae sp. 69	KP410723
Ostracoda	Podocopa	Podocopida	Bairdiidae sp. 4	KP410726
Ostracoda	Podocopa	Podocopida	Bairdiidae sp. 5	KP734272
Ostracoda	Podocopa	Podocopida	Bairdiidae sp. 14	KP734273
Ostracoda	Podocopa	Podocopida	Bairdiidae sp. 41	KP410728
Ostracoda	Podocopa	Podocopida	Bairdiidae sp. 59	KP734274
Ostracoda	Podocopa	Podocopida	Bairdiidae sp. 60	KP734275
Ostracoda	Podocopa	Podocopida	Pontocyprididae sp. 7	KP734276
Ostracoda	Podocopa	Podocopida	Pontocyprididae sp. 24–2	KP734277
Ostracoda	Podocopa	Podocopida	Pontocyprididae sp. 26–1	KP734278
Ostracoda	Podocopa	Podocopida	Pontocyprididae sp. 26–2	KP734279
Ostracoda	Podocopa	Podocopida	Pontocyprididae sp. 26–3	KP734280
Ostracoda	Podocopa	Podocopida	Macrocyprididae sp. 2–1	KP734282
Ostracoda	Podocopa	Podocopida	Macrocyprididae sp. 2–2	KP734283
Ostracoda	Podocopa	Podocopida	Macrocyprididae sp. 2–3	KP734284
Ostracoda	Podocopa	Podocopida	Xestoleberididae sp. 18	KP410727
Ostracoda	Podocopa	Podocopida	Xestoleberididae sp. 44	KP734285
Ostracoda	Podocopa	Podocopida	Xestoleberididae sp. 55	KP734286
Ostracoda	Podocopa	Podocopida	Cytheromatidae sp. 40	KP734288
Ostracoda	Podocopa	Platycopida	Cytherellidae sp. 54	KP734290
Ostracoda	Podocopa	Podocopida	Cytheroidea sp. 52	KP734289
Ostracoda	Podocopa	Podocopida	Loxoconchidae sp. 21	KP734291
Ostracoda	Podocopa	Podocopida	Paradoxostomatidae sp. 30	KP734287

Numbers after species name indicate sample identifiers

Table 2 Species and GenBank accession numbers of 28S rDNA sequences (amplified with dd-ff primers) used in this study

Class	Subclass	Order	Species	GenBank No.
Ostracoda	Podocopa	Podocopida	Bairdiidae sp. 5	KP793225
Ostracoda	Podocopa	Podocopida	Bairdiidae sp. 4	KP793226
Ostracoda	Podocopa	Podocopida	Bairdiidae sp. 14	KP793227
Ostracoda	Podocopa	Podocopida	Bairdiidae sp. 60	KP793228
Ostracoda	Podocopa	Podocopida	Macrocyprididae sp. 2–1	KP793231
Ostracoda	Podocopa	Podocopida	Macrocyprididae sp. 2–2	KP793232
Ostracoda	Podocopa	Podocopida	Macrocyprididae sp. 2–3	KP793233
Ostracoda	Podocopa	Podocopida	Xestoleberididae sp. 55	KP793229
Ostracoda	Podocopa	Podocopida	Xestoleberididae sp. 44	KP793230

Numbers after species name indicate sample identifiers

to superfamily or family level based mostly on external carapace morphology. Myodocopa was represented by six families: Cylindroleberididae, Philomedidae, Sarsiellidae, Rutidermatidae, Cypridinidae Baird, 1850, and Polycopidae Sars, 1865. The Podocopa was represented by three cytheroidean families (Xestoleberididae, Loxoconchidae, and Cytheromatidae) and the families Bairdiidae, Macrocyprididae, Pontocyprididae, Cytherellidae, and Candonidae Kaufmann, 1900 (subfamily Paracypridinae Sars, 1923). Sarsiellidae was the most representative family of the subclass Myodocopa with 122 individuals and 16 species and for Podocopa, Bairdiidae showed the highest number of individuals and species (728 and 13, respectively) (Figs. 2 and 3). Photographs of selected species of Myodocopa and Podocopa from each identified family are provided in Fig. 4.

Ostracods were highly diverse and regularly found in the substrata of Caribbean mesophotic coral ecosystems (MCEs) at depths between 30 and 102 m. The highest densities of ostracods were located in the deepest samples (≥61 m) and these were the most abundant and diverse assemblages, second only to harpacticoid copepods. The deepest sampling sites yielded three times more individuals (average $n = 65$) compared to the number of specimens recovered from shallow and intermediate depths (Fig. 5). Moreover, the number of species increased with depth and the deepest sampling sites showed the highest species number (total $n = 72$) (Fig. 5). The most abundant subclass in shallow depths was the Myodocopa with 272 individuals; however, Podocopa was the most abundant subclass in intermediate and deeper sampling locations (Fig. 6a). The deepest samples were highly represented by podocopans (1298 specimens) (Fig. 6a). Similarly, a clear pattern of species number with depth is observed. In both subclasses the number of species increased with depth and the deepest sampling sites showed the highest number of species (Fig. 6b). The Podocopa was also the most diverse subclass in terms of species compared to the subclass Myodocopa (Fig. 6b). The highest relative abundance of ostracods collected from MCEs of Puerto Rico was observed in Vieques (72 %) followed by Tourmaline (10 %) and the stations of Ponce and Bajo de Sico both with 4 % (Fig. 7). The highest numbers of species were found in the Puerto Rican localities of La Parguera (total $n = 43$) and Vieques (total $n = 34$) (Fig. 8).

Molecular barcoding

The pairwise comparison between 28S sequences (amplified with v-x primers) for selected species of the subclass Myodocopa showed that the highest estimated values of evolutionary divergence were observed between families, as expected; the highest value (9.3 %) was observed between sp. 11 (Rutidermatidae) and sp. 66 (Cylindroleberididae); the same value was also observed when comparing sp. 68 (Rutidermatidae) and sp. 66 (Cylindroleberididae) (Table 3, Fig. 9). Smaller sequence divergences were observed between species of the same family; the lowest value (0.4 %) was observed between sp. 11 and sp. 68, both species belong to the family Rutidermatidae (Table 3, Fig. 9). The vast majority of morphologically identified specimens as different species yielded different sequences; therefore, presumably, almost all separated specimens belonged to different species. Our sampling was limited to U.S. Caribbean; therefore, we might have missed lineages of ostracods outside this region. Partial sampling of lineages may lead to an overestimation of species because the true extent of within species variation is unknown. We limited this potential problem by choosing a conservative

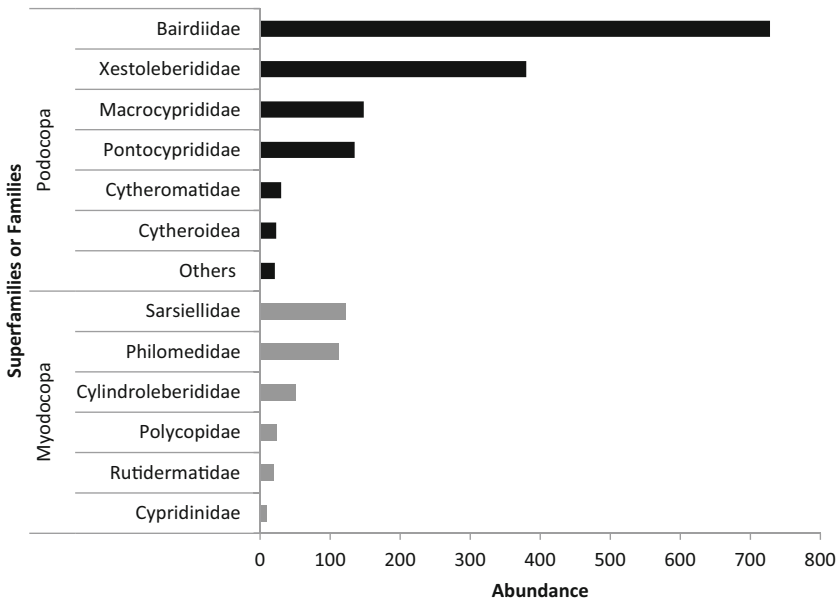

Fig. 2 Total abundance of ostracods between superfamilies or families of the subclasses Myodocopa and Podocopa from all mesophotic sampling sites. Others=Loxoconchidae, Cytherellidae, and Candonidae

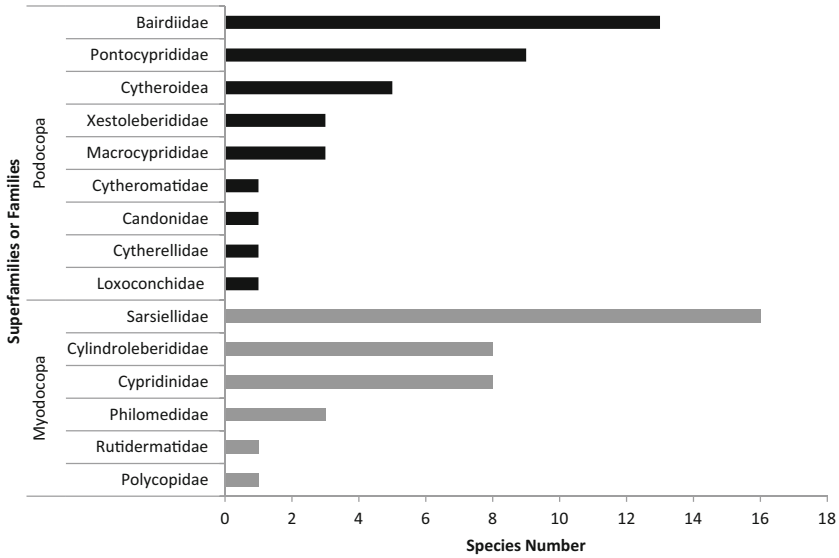

Fig. 3 Number of ostracod species between superfamilies or families of the subclasses Podocopa and Myodocopa from all mesophotic sampling sites

region of 28S. Phylogenetic analysis supported monophyly of some families of Myodocopa (Fig. 10).

The highest values of sequence divergence for selected species of Podocopa were also observed between superfamilies (Cytheroidea) or families (Table 4). Species of the same family showed the lowest rates of divergence; the highest value (14.9 %) was observed between sp. 44 (Xestoleberididae) and sp. 26–3 (Pontocyprididae) (Table 4, Fig. 11). The lowest sequence divergence (0.2 %) was observed between two species of the family Bairdiidae (sp. 4 and sp. 5) (Table 4, Fig. 11). All morphologically identified Podocopa species yielded different sequences. Moreover, the results from the phylogenetic analysis follow the same pattern as those in Myodocopa; most superfamilies or families of Podocopa formed monophyletic groups (Fig. 10). An additional region of the 28S rDNA gene was also amplified with primers dd-ff for selected species of the families Bairdiidae, Xestoleberididae, and Macrocyprididae since the external morphology (carapace) was extremely similar between specimens of the same family or yielded identical sequences with the v-x primers (Fig. 12). The pairwise comparison showed similar results; the highest estimates values of evolutionary

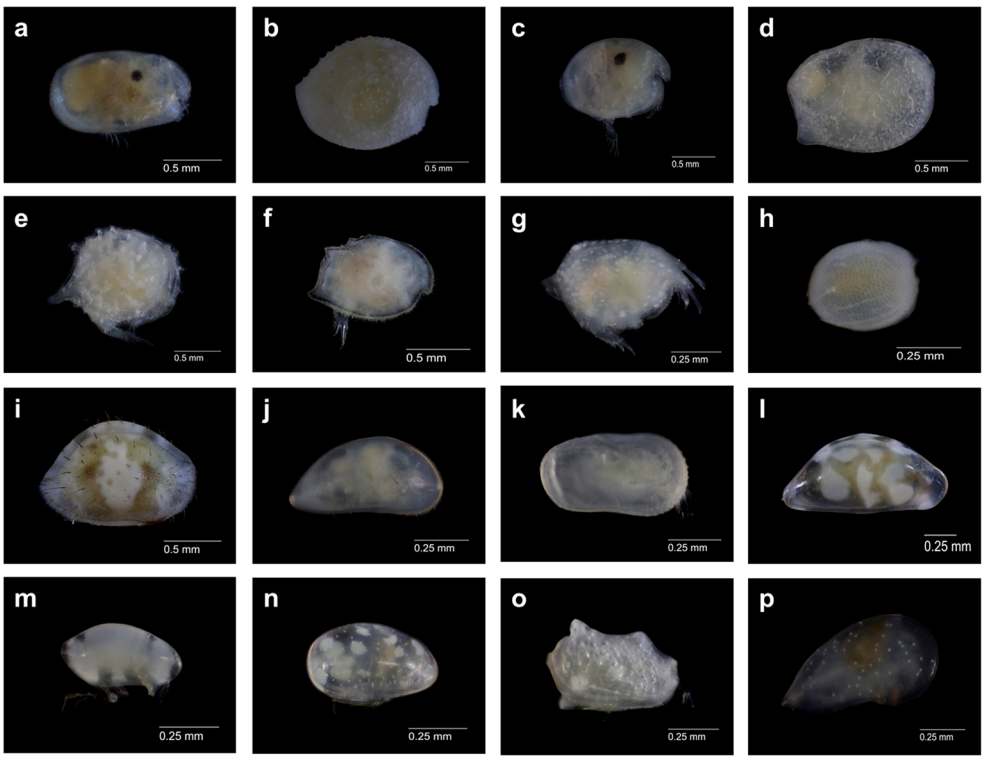

Fig. 4 Photographs showing selected ostracods from the representative families of the subclass Myodocopa: (**a**) Cylindroleberididae sp. 22–1, (**b**) Cylindroleberididae sp. 48, (**c**) Cypridinidae sp. 29–2, (**d**) Sarsiellidae sp. 51, (**e**) Sarsiellidae sp. 45, (**f**) Rutidermatidae sp. 11, (**g**) Philomedidae sp. 9, and (**h**) Polycopidae sp. 42 and the subclass Podocopa: (**i**) Bairdiidae sp. 4, (**j**) Pontocyprididae sp. 26–2, (**k**) Cytherellidae sp. 54, (**l**) Macrocyprididae sp. 2–1, (**m**) Cytheromatidae sp. 40, (**n**) Xestoleberididae sp. 55, (**o**) Loxoconchidae sp. 76, and (**p**) Candonidae sp. 38. All photographs were taken by the first author with a confocal microscope (Olympus FV300)

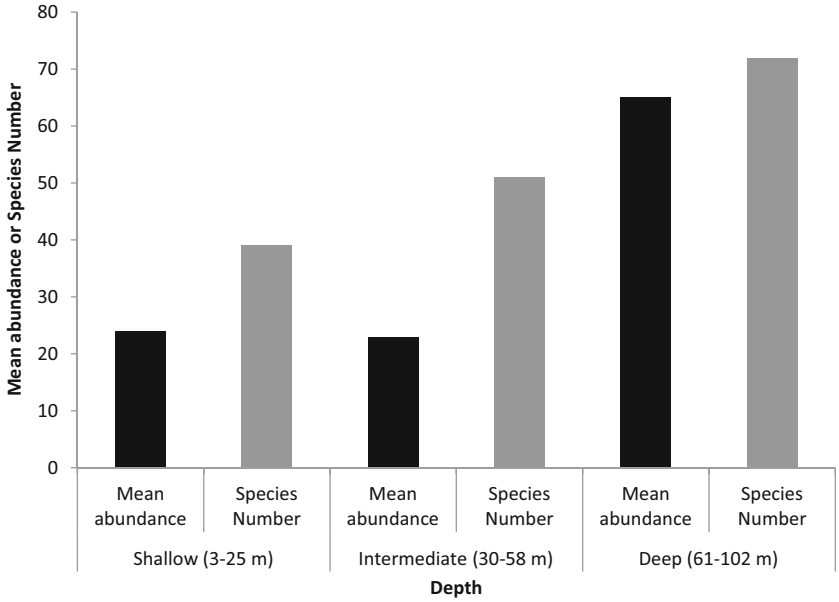

Fig. 5 Mean abundance and number of ostracod species found at each depth range in all sampling sites

divergence for selected species of Podocopa were observed between families and species of the same family showed the lowest divergence estimates (Table 5). However sp. 44 and 44–1 showed 0 % divergence suggesting that both specimens

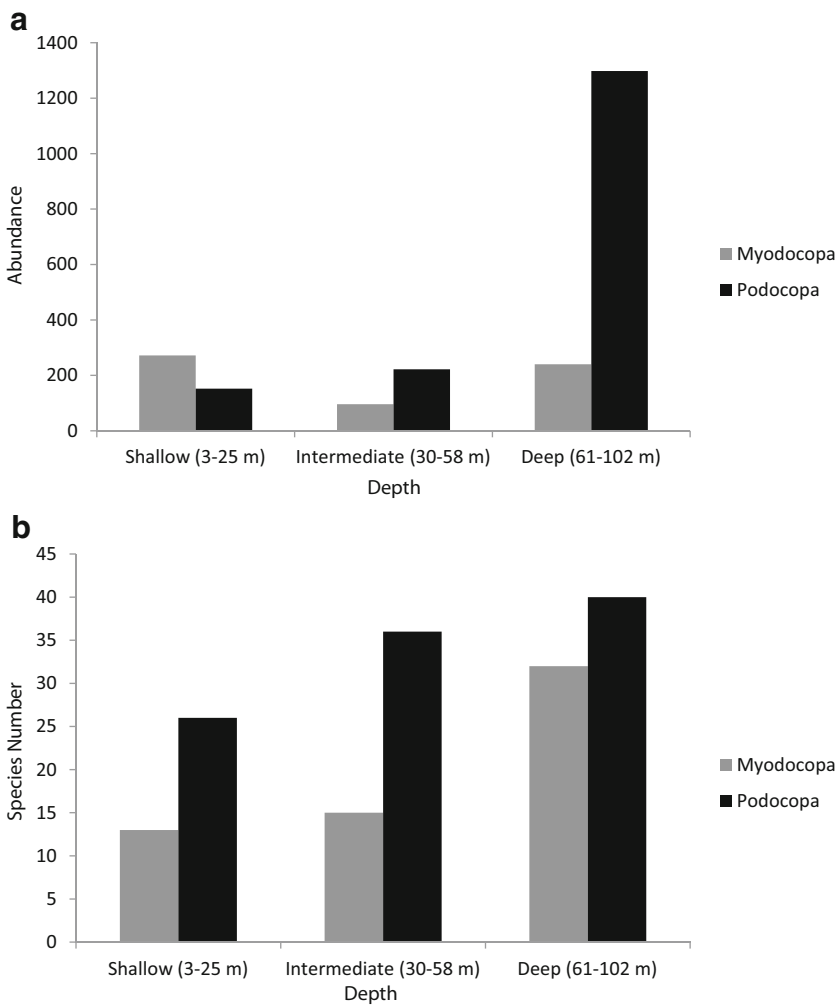

Fig. 6 Total abundance (**a**) and number of ostracod species (**b**) by subclass at each depth range from all sampling sites

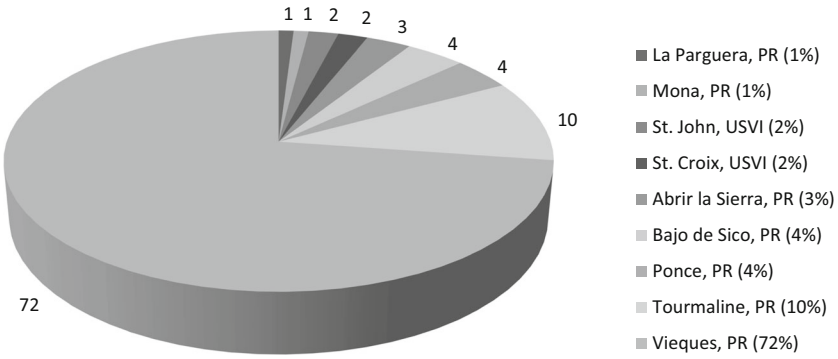

Fig. 7 Relative abundance (%) of ostracods at each mesophotic sampling site

belong to the same species (Fig. 13). Alternatively, if these specimens belong to different species, they exhibit no differentiation in this portion of 28S. The phylogenetic analysis of Podocopa supported the monophyly of Xestoleberididae and Macrocyprididae; the Bairdiidae formed another group (Fig. 14).

Discussion

Biological

The marine ostracod fauna is considered to be a warm temperature fauna typically found in tropical and subtropical regions (Kornicker 1961; Baker and Hulings 1966; Kornicker 1979); for this reason Puerto Rico, the U.S. Virgin Islands, and generally the Caribbean region provide a suitable environment for the development of a highly speciose ostracod community. Previous studies of ostracods have shown that the substrate type, as well as the physical and chemical properties of the specific habitat, is an important factor influencing the distribution of most species (Kornicker 1979; Ruiz et al. 1994; Alvarez et al. 2000). Benthic ostracods are a substantial component of meiofaunal communities, and one of the most important determinants of these communities is the grain size (McIntyre 1969). Snelgrove (1998) mentioned that the distribution of benthic communities is closely related to the following factors: differences in sediment type, temperature, salinity, primary productivity, depth, physical disturbance, and historical disturbance. Therefore, a combination of these factors, particularly the substrate composition could explain the highest yielding of abundance and diversity in Vieques. Vieques is located within the Puerto Rico-Virgin Island geological platform in the northeastern Caribbean. For many years, the island was used for military activities and consequently most of the area was restricted to the public and the access for scientific research was very limited (Rivero-Calle et al. 2008). Recent studies of Vieques have shown that the coral reef communities are healthy and considered among the best in Puerto Rico in terms of live coral coverage (>30 %), specifically the deeper mesophotic reefs (30–100 m) that are not impacted directly by hurricanes, changes in water temperatures, or military debris (García-Sais et al. 2001, 2004; Rivero-Calle et al. 2008; García-Sais et al. 2011). At depths of 30–100 m, where MCEs are found, the benthos of the southern part of Vieques is characterized in general by the presence of unconsolidated sediment with encrusting algae and cyanobacteria, rhodolith beds, sponges, gorgonians,

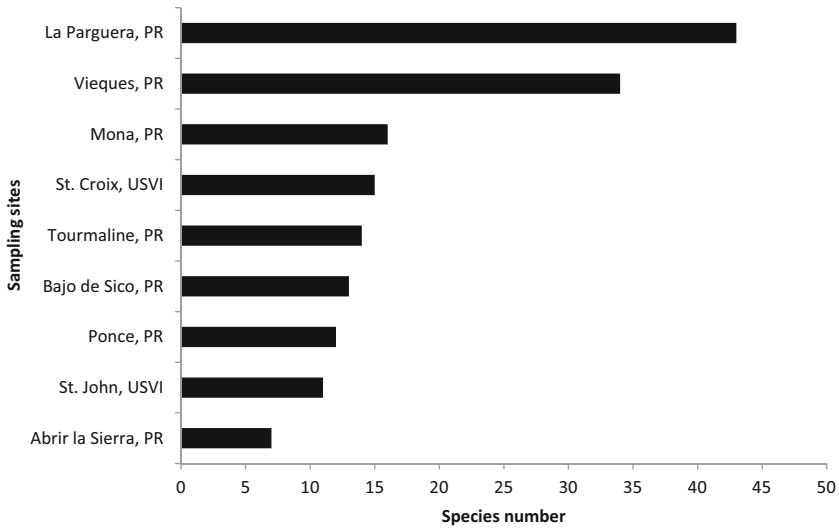

Fig. 8 Number of ostracod species at each mesophotic sampling site

Table 3 Uncorrected pairwise genetic distance of selected Myodocopa based on 568 bp of 28S rDNA sequences (amplified with v-x primers)

	1	2	3	4	5	6	7	8	9	10
1Cylindroleberididae sp. 22–2										
2Cylindroleberididae sp. 22–3	0.057									
3Cylindroleberididae sp. 66	0.059	0.018								
4Cylindroleberididae sp. 67	0.060	0.053	0.055							
5Sarsiellidae sp. 8	0.083	0.080	0.081	0.078						
6Sarsiellidae sp. 51	0.076	0.080	0.083	0.074	0.013					
7Rutidermatidae sp. 11	0.083	0.087	0.093	0.080	0.032	0.035				
8Rutidermatidae sp. 68	0.083	0.091	0.093	0.076	0.032	0.035	0.004			
9Philomedidae sp. 58	0.087	0.091	0.091	0.083	0.028	0.030	0.039	0.035		
10Philomedidae sp. 69	0.077	0.083	0.085	0.079	0.048	0.044	0.037	0.039	0.057	

The number of base differences per site from between sequences are shown. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.5). The analysis involved ten nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (Kumar et al. 2016)

corals, sand, and fine sediment (Rivero-Calle et al. 2008). The optimum state of these ecosystems might be as well a major factor that influenced the high abundance and diversity of the benthic ostracod community associated with MCEs.

In contrast, one of the most impacted mesophotic sites, Ponce has a narrow insular shelf and hosts several river outlets, a commercial port, a regional sewage treatment plant with associated deep water outfall, and three deep dredge disposal sites (Appeldoorn et al. 2015). MCEs of Ponce receive higher (16x) rates of sedimentation and enriched ^{15}N (an indicator of sewage) concentrations than off La Parguera, a less impacted site. The relative abundance of ostracods in the Ponce station was much lower compared to Vieques, but it was higher than La Parguera suggesting that other factors besides levels of impact also control the ostracod communities (Fig. 7).

Another important factor associated with the distribution and diversification of ostracods is depth. According to Kornicker (1961), earlier studies by Remane (1933), Elofson (1941), and Benson (1959) have been focused on depth zonation of ostracods. The zonation is associated primarily with changes in environmental factors such as temperature, food source, and current velocities. Studies of the genus *Bairdia* M'Coy, 1844 (Podocopa) indicated that several species are limited to a specific depth range (Kornicker 1961). Our results revealed that the number of species increased with depth (Figs. 5 and 6b). The highest diversity of ostracods was found at the deepest sampling stations (61–102 m) and the lowest at the shallow stations (3–25 m). These results suggest that the environmental conditions in the MCEs at depths ≥61 m are appropriate for the establishment of highly diversified ostracod communities. We observed the highest abundance of ostracods in the deepest sampling stations (61–102 m) (Figs. 5 and 6a). Nevertheless, our study was not designed to test the relationship between depth and the abundance of species in Puerto Rico.

The subclass Podocopa has a wide range distribution, and many species have been reported worldwide, including the Caribbean region. Podocopid ostracods are benthic, living as infaunal burrowers, epifaunal crawlers, or demersal swimmers (Maddocks et al. 2007). Our results showed that the Podocopa was the most representative group of Caribbean MCEs with the highest abundance and diversity. The Bairdiidae was the most numerically dominant podocopan family with 728 individuals represented by 13 species (Figs. 2 and 3). Species belonging to this family can be found in shallow and deep waters ranging from 0.6 to more than 3000 m (Puri and Hulings 1957; Hulings 1959; Kornicker 1961; Baker and Hulings 1966; Maddocks 1969; Maddocks et al. 2007) with

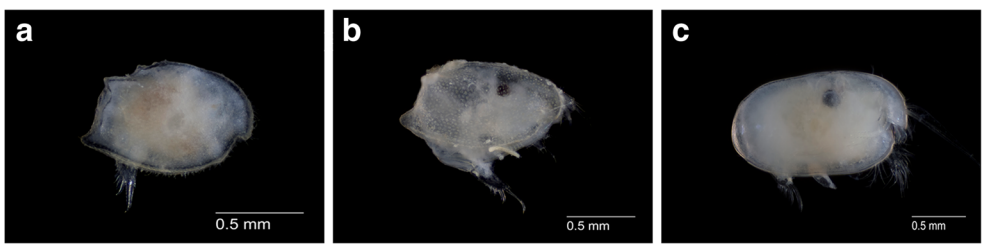

Fig. 9 Photographs of myodocopan ostracods that showed the highest (9.3 %) or lowest (0.4 %) sequence divergence in the pairwise comparison for the 28S (v-x) region: (**a**) Rutidermatidae sp. 11, (**b**) Rutidermatidae sp. 68, and (**c**) Cylindroleberididae sp. 66. All photographs were taken by the first author with a confocal microscope (Olympus FV300)

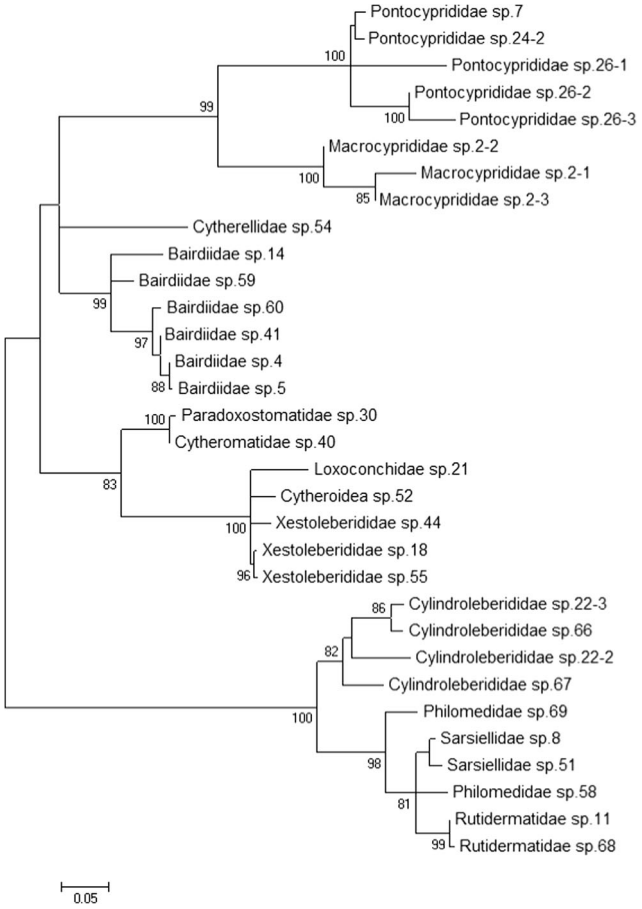

Fig. 10 Maximum Likelihood tree of selected Myodocopa and Podocopa based on 28S rDNA (v-x) sequence data (568 bp). The bootstrap consensus tree was inferred from 1000 replicates. Numbers above branches indicate bootstrap support. Branches without numbers reproduced in <75 % bootstrap replicates (Kimura 1980; Kumar et al. 2016)

some species being restricted to certain depths. Even though the highest densities of bairdiids were found between 30 and 102 m, specimens were also found in shallow samples from 3 to 25 m. According to Kornicker (1961), bairdiid ostracods are not limited to a narrow temperature range but the presence of large densities reflects warm waters; our results confirm that Bairdiidae are abundant and highly diverse in tropical waters.

The subclass Myodocopa has also a worldwide distribution, and species have been reported from the Caribbean region, including two recently described new species for Puerto Rico from shallow waters: *Diasterope puertoricensis* Morales-Núñez & Kornicker, 2007 (Cylindroleberididae) and *Euphilomedes chupacabra* Lum et al., 2008 (Philomedidae). This group has been extensively studied by Kornicker (1981, 1983, 1984a, b, 1986a, b). Myodocopid ostracods are exclusively marine and mostly benthic and include all planktonic species (Kornicker 1979; Cohen 1982; Ruppert et al. 2004). Although the total number of myodocopid specimens (total $n = 339$) collected from MCEs was less compared to the podocopids, they were found in most of the sampled locations. Specimens of all the extant families of the order Myodocopida Sars, 1866 were identified, with the Sarsiellidae and Philomedidae being the most abundant; in addition specimens of the family Polycopidae (order Halocyprida Dana, 1853) were also identified (Fig. 2). Besides the low numbers of individuals within families compared to some superfamilies or families of Podocopa, our results showed that myodocopids were also highly diverse in terms of species. Species of Myodocopa can be found from the surface to abyssal depths (Cohen 1982), and according to our results, the highest densities were found in both shallow (3–25 m) and deep (61–102 m) depths (Fig. 6a). The presence of myodocopids in Caribbean MCEs reflects again the considerable influence of water temperature on the distribution of many ostracods species.

Molecular barcoding

DNA barcoding has been a useful method to facilitate species-level identification analysis and global biodiversity assessment (Hebert et al. 2003, 2004; Hajibabaei et al. 2006, 2007; Bucklin et al. 2011). In the present work we used a ~580 to ~900 base-pair regions (28S v-x & 28S dd-ff) of the nuclear large subunit ribosomal DNA (28S rDNA) for species recognition of selected specimens of Ostracoda. Because of their small size (0.2–2 mm) and the fact that many species are sexually dimorphic, identification to lower taxonomic levels is a major challenge for non-specialists. Sexual dimorphism can be observed in the internal soft anatomy including testes, ovaries, and associated structures, external genitalia, appendages, domicilum (brooders), and external size, shape, and carapace characteristics (Cohen et al. 2007). The morphological characterization of specimens of Myodocopa and Podocopa was based mostly on external carapace morphology; therefore, we applied molecular barcoding to discriminate between species at the molecular level, thus supporting the morphological identifications.

The results revealed the usefulness of 28S rDNA regions as appropriate markers for phylogenetic questions at different taxonomic levels and their efficiency in the identification of ostracods at the species level. The pairwise comparisons (sequences amplified with the v-x primers) for Myodocopa showed a high evolutionary divergence between species of different families, but lower divergence between species of the same family, as expected (Table 3). Phylogenetic analysis by the Maximum Likelihood (ML) method supported the monophyly of most families of Myodocopa; the Sarsiellidae, Philomedidae, and Rutidermatidae formed a distinct clade (bootstrap support 98 %) and the cylindroleberidids formed another group (bootstrap support 82 %) (Fig. 10). Similar results were observed for the Podocopa (sequences amplified with the v-x primers); between families the evolutionary

Table 4 Pairwise genetic distance of selected Podocopa based on 568 bp of 28S rDNA sequences (amplified with v-x primers)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1Bairdiidae sp. 4																						
2Bairdiidae sp.5	0.002																					
3Bairdiidae sp. 14	0.044	0.044																				
4Bairdiidae sp. 41	0.006	0.008	0.038																			
5Bairdiidae sp. 59	0.042	0.044	0.036	0.036																		
6Bairdiidae sp. 60	0.015	0.013	0.036	0.010	0.036																	
7Pontocyprididae sp. 7	0.104	0.104	0.119	0.110	0.118	0.110																
8Pontocyprididae sp. 24–2	0.108	0.108	0.125	0.113	0.125	0.113	0.012															
9Pontocyprididae sp. 26–1	0.114	0.114	0.123	0.119	0.121	0.119	0.033	0.055														
10Pontocyprididae sp. 26–2	0.116	0.116	0.119	0.121	0.125	0.119	0.041	0.032	0.069													
11Pontocyprididae sp. 26–3	0.121	0.121	0.125	0.127	0.129	0.125	0.041	0.053	0.069	0.024												
12Macrocyprididae sp. 2–1	0.106	0.108	0.110	0.104	0.100	0.106	0.089	0.116	0.089	0.113	0.114											
13Macrocyprididae sp. 2–2	0.102	0.104	0.110	0.104	0.102	0.106	0.091	0.091	0.110	0.090	0.112	0.032										
14Macrocyprididae sp. 2–3	0.100	0.102	0.106	0.102	0.098	0.104	0.088	0.114	0.110	0.113	0.091	0.026	0.028									
15 Xestoleberididae sp. 44	0.115	0.113	0.115	0.117	0.102	0.119	0.125	0.127	0.129	0.145	0.149	0.147	0.143	0.147								
16 Xestoleberididae sp. 18	0.109	0.107	0.115	0.111	0.096	0.113	0.130	0.131	0.133	0.143	0.147	0.137	0.133	0.137	0.015							
17 Xestoleberididae sp. 55	0.113	0.111	0.115	0.115	0.100	0.117	0.125	0.127	0.129	0.139	0.143	0.141	0.137	0.141	0.015	0.004						
18Paradoxostomatidae sp. 30	0.098	0.100	0.096	0.096	0.081	0.100	0.111	0.117	0.117	0.121	0.123	0.113	0.117	0.117	0.077	0.072	0.072					
19Cytheromatidae sp. 40	0.096	0.098	0.094	0.094	0.079	0.098	0.111	0.117	0.115	0.119	0.121	0.111	0.115	0.115	0.074	0.068	0.068	0.004				
20Cytheroidea sp. 52	0.115	0.113	0.121	0.117	0.105	0.119	0.125	0.125	0.129	0.139	0.141	0.141	0.135	0.137	0.021	0.015	0.017	0.077	0.074			
21Cytherellidae sp. 54	0.086	0.088	0.091	0.080	0.071	0.082	0.108	0.119	0.114	0.131	0.135	0.110	0.114	0.110	0.109	0.102	0.107	0.102	0.098	0.113		
22Loxoconchidae sp. 21	0.105	0.107	0.103	0.107	0.095	0.109	0.121	0.127	0.127	0.140	0.144	0.133	0.131	0.133	0.032	0.034	0.030	0.074	0.074	0.040	0.109	

The number of base differences per site from between sequences are shown. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.5). The analysis involved 22 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (Kumar et al. 2016)

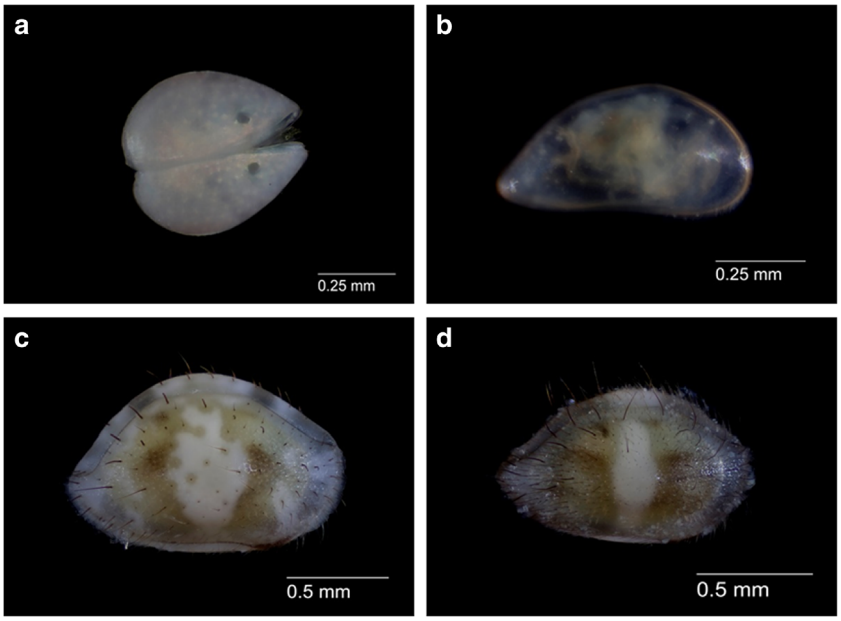

Fig. 11 Photographs of podocopan ostracods that showed the highest (14.9 %) or lowest (0.2 %) sequence divergence in the pairwise comparison for the 28S (v-x) region: (**a**) Xestoleberididae sp. 44, (**b**) Pontocyprididae sp. 26–3, (**c**) Bairdiidae sp. 4, and (**d**) Bairdiidae sp. 5. All photographs were taken by the first author with a confocal microscope (Olympus FV300)

divergence of species was higher than species belonging to the same family (Table 4). In addition, the phylogenetic analysis by ML method showed the same trend; most families of Podocopa formed a distinct monophyletic clade (Fig. 10). Similarly, the same pattern was also observed in the pairwise comparison and ML method between selected species of the

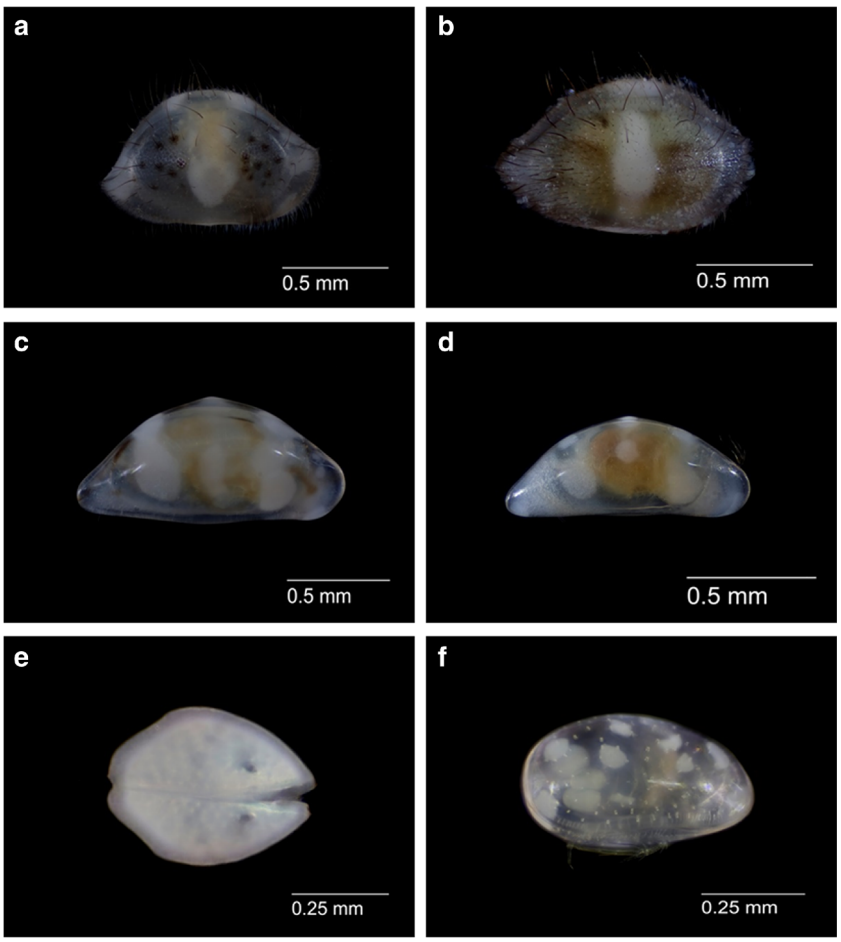

Fig. 12 Photographs of some of the selected podocopan species of the families Bairdiidae, Xestoleberididae and Macrocyprididae used for the pairwise comparison of the 28S (dd-ff) region: (**a**) Bairdiidae sp. 14, (**b**) Bairdiidae sp. 5, (**c**) Macrocyprididae sp. 2–3, (**d**) Macrocyprididae sp. 2–2, (**e**) Xestoleberididae sp. 44–1, and (**f**) Xestoleberididae sp. 55. All photographs were taken by the first author with a confocal microscope (Olympus FV300)

Table 5 Pairwise genetic distance of selected Podocopa based on 651 bp of 28S rDNA sequences (amplified with dd-ff primers)

	1	2	3	4	5	6	7	8	9	10
1Bairdiidae sp. 5										
2Bairdiidae sp. 4	0.005									
3Bairdiidae sp. 14	0.071	0.064								
4Bairdiidae sp. 60	0.068	0.063	0.049							
5Macrocyprididae sp. 2–1	0.141	0.145	0.134	0.143						
6Macrocyprididae sp. 2–2	0.145	0.149	0.138	0.147	0.026					
7Macrocyprididae sp. 2–3	0.139	0.143	0.132	0.141	0.026	0.026				
8Xestoleberididae sp. 55	0.108	0.108	0.112	0.117	0.162	0.166	0.161			
9Xestoleberididae sp. 44	0.103	0.103	0.109	0.109	0.165	0.168	0.166	0.049		
10Xestoleberididae sp. 44–1	0.103	0.103	0.109	0.109	0.165	0.168	0.166	0.049	0.000	

The number of base differences per site from between sequences are shown. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.1). The analysis involved ten nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (Kumar et al. 2016)

families Bairdiidae, Macrocyprididae, and Xestoleberididae amplified with the dd-ff primers (Table 5, Fig. 14).

These results supported our hypothesis that all morphologically defined species are also different at the molecular level testing the concordance between morphological vs. genetic species concepts with this portion of 28S. The molecular analyses demonstrated that the assignment of different species determined mainly by the morphology of external anatomy (carapace) to corresponding families of Ostracoda was robust. A potential caveat to this approach is that in the instances where the sequence divergent is small (<2 %), it is possible that we have observed within-species sequence divergence. Furthermore, in these particular cases where the external morphology between specimens was almost identical or we suspected that species were sexually dimorphic even though they presented a slight difference in carapace morphology the amplification of the additional 28S dd-ff region helped to corroborate the 28S v-x results, thus supporting the efficiency of the 28S marker. On the other hand, the nuclear ribosomal genes are under the influence of concerted evolution, which homogenizes gene copy variants (Hillis and Dixon 1991). A fast rate of sequence homogenization will result in no or very low intraspecific variability within these genes; therefore, even single substitutions may indicate closely related but distinct (and in many cases cryptic) species.

Even though identification and description of new species are accomplished by detailed taxonomic work, the use of DNA barcodes has facilitated the overall process; in other words, DNA barcoding complements taxonomy (Hajibabaei et al. 2007). Bucklin et al. (2011) also pointed out that DNA barcodes are especially useful for identification of rare, fragile, and/or small species when identification based on morphology is difficult. The Ostracoda has been an ideal group for

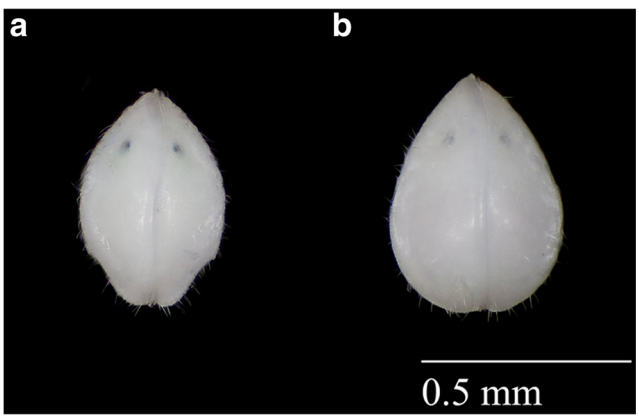

Fig. 13 Photographs of the morphotypes of sp. 44 (Xestoleberididae) designated as sp. 44–1 (**a**) and sp. 44 (**b**) that showed 0 % in the pairwise comparison of the 28S (dd-ff) region suggesting that both specimens belong to the same species. We suspect that the species present sexual dimorphism. The photograph was taken by José Almodóvar (UPRM) with a confocal microscope (Olympus FV300)

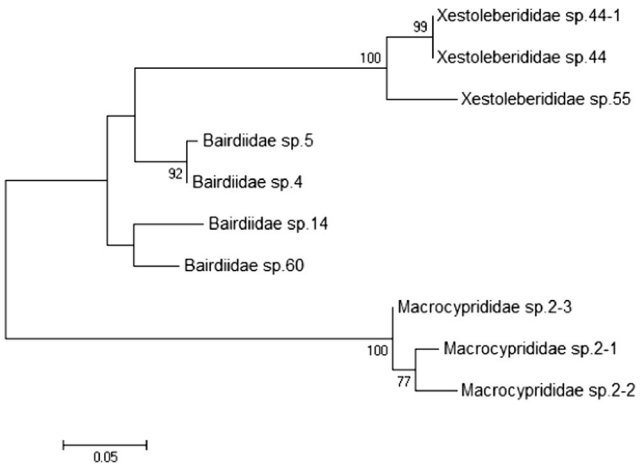

Fig. 14 Maximum Likelihood tree of selected Podocopa based on 28S rDNA (dd-ff) sequence data (651 bp). The bootstrap consensus tree was inferred from 1000 replicates. Numbers above branches indicate bootstrap support. Branches without numbers reproduced in <75 % bootstrap replicates (Kimura 1980; Kumar et al. 2016)

DNA barcoding studies specifically at species level identification, in this case using a region of the 28S rDNA. Previous works by Oakley and Cunningham (2002) and Syme and Oakley (2012) used the same marker to study eye evolution in myodocopid ostracods. Other markers commonly used for phylogenetic studies of ostracods include 16S rDNA, 18S rDNA, and mitochondrial cytochrome *c* oxidase subunit 1 (*CO1*) (Oakley and Cunningham 2002; Ogoh and Ohmiya 2004; Yamaguchi and Endo 2003; Wakayama and Abe 2006; Tinn and Oakley 2008).

This study provides the first baseline characterization of ostracods from MCEs of the Caribbean region, especially Puerto Rico and U.S. Virgin Islands. Appreciation of the true levels of biodiversity, especially the small macrofauna and meiofauna inhabiting mesophotic reefs will enhance decisions of policy-making agencies managing these near shore resources. Taxonomically centered studies coupled with genetic approaches will improve our understanding of the evolution, ecology, and zoogeographical distribution patterns of mesophotic ostracods.

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