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Species boundaries of Gulf of Mexico vestimentiferans (Polychaeta, Siboglinidae) inferred from mitochondrial genes

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At least six morphospecies of vestimentiferan tubeworms are associated with cold seeps in the Gulf of Mexico (GOM). The physiology and ecology of the two best-studied species from depths above 1000 m in the upper Louisiana slope (Lamellibrachia luymesi and Seepiophila jonesi) are relatively well understood. The biology of one rare species from the upper slope (escarpiid sp. nov.) and three morphospecies found at greater depths in the GOM (Lamellibrachia sp. 1, L. sp. 2, and Escarpia laminata) are not as well understood. Here we address species distributions and boundaries of cold-seep tubeworms using phylogenetic hypotheses based on two mitochondrial genes. Fragments of the mitochondrial large ribosomal subunit rDNA (16S) and cytochrome oxidase subunit I (COI) genes were sequenced for 167 vestimentiferans collected from the GOM and analyzed in the context of other seep vestimentiferans for which sequence data were available. The analysis supported five monophyletic clades of vestimentiferans in the GOM. Intra-clade variation in both genes was very low, and there was no apparent correlation between the within-clade diversity and collection depth or location. Two of the morphospecies of Lamellibrachia from different depths in the GOM could not be distinguished by either mitochondrial gene. Similarly, E. laminata could not be distinguished from other described species of Escarpia from either the west coast of Africa or the eastern Pacific using COI. We suggest that the mitochondrial COI and 16S genes have little utility as barcoding markers for seep vestimentiferan tubeworms.

1. Introduction

For the better part of the last century, marine biologists assumed oceans were largely interconnected by currents that enabled larvae and propagules to reach distant shores and assure gene flow even over great distances. More recently, the use of molecular tools has challenged assumptions regarding population structure and speciation in the ocean and demonstrated that marine animals often have genetically distinct populations despite geographic proximity (Palumbi and Warner, 2003). Although sharp genetic breaks between close populations have been recorded throughout the ocean, most of what is known about speciation patterns and phylogeography has been inferred from shallow-water and coastal systems, which represent only about 15% of the aquatic environment. Thus, our knowledge of processes that lead to population divergence and speciation in the open ocean is relatively limited (Thornhill et al., 2008, and references therein; Zardus et al., 2006).

Vestimentiferan tubeworms, which include 10 genera in the polychaete family Siboglinidae (Halanych et al., 2001; Kojima et al., 2002; McMullin et al., 2003; Rouse, 2001), are abundant at deep-sea hydrothermal vents and cold seeps at depths ranging from 80 to 9345 m (Cordes et al., 2007b; Mironov, 2000; Miura et al., 2002). In the deep Gulf of Mexico, six morphospecies have been reported (Cordes et al., 2009). Two described species, Lamellibrachia luymesi (van der Land and Narrevang, 1975) and Seepiophila jonesi (Gardiner et al., 2001), are relatively well studied, and their ecology and physiology are well understood (Bergquist et al., 2002; Cordes et al., 2007a, b). They occur on the upper Louisiana slope at between ~500 and 950 m depth and occasionally co-occur with a rare undescribed species, escarpiid sp. nov. The three other morphological species are found on the lower Louisiana slope at depths greater than about 950 m (Lamellibrachia sp. 1, L. sp. 2, and Escarpia laminata).

In this paper, we present phylogenetic hypotheses based on the mitochondrial large ribosomal subunit rDNA gene (16S) and mitochondrial cytochrome oxidase 1 gene (COI) of over 200

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vestimentiferans (sequenced for either or both genes) including 180 individuals from the six morphospecies that occur in the Gulf of Mexico. Phylogenetic trees are used to examine the distribution of vestimentiferans in the Gulf of Mexico and their relations to other vestimentiferans around the world. We examined the concordance between the morphological and phylogenetic data to identify differences between the genealogical and morphological species analyzed. Finally, we compared between- and within-species 16S and COI genetic distances and show that these two mitochondrial genes have little utility as “barcoding molecules” for vestimentiferans.

2. Material and methods

2.1. Collection of material

Vestimentiferans were collected in the deep Gulf of Mexico from 12 sites on two cruises in 2006 and 2007, using the DSV ALVIN and R.V. Atlantis in 2006 and ROV JASON II and the NOAA ship Ronald Brown in 2007 (see Fig. 1). Vestimentiferans were collected using either the Bushmaster Jr. collection device (for samples destined also for community ecology analyses, see Cordes et al., 2010) or the submersible manipulators and placed directly into a collection box. Aboardship, all vestimentiferans were identified using morphological criteria, and subsamples of vestimentum tissue were frozen for subsequent analyses at the Pennsylvania State University. Additional frozen vestimentiferan tissue samples collected previously from shallower sites on the upper Louisiana slope using the DSV JOHNSON SEA LINK were also analyzed for this study (see Table 1 for a complete list of specimens).

2.2. DNA sequencing

DNA was extracted either by boiling a small amount of frozen tissue in 600 µL of 10% Chelex solution (Bio-Rad) or using a CTAB+PVP method modified from Doyle and Doyle (1987), followed by a standard ethanol precipitation.

A 524 bp fragment of the mitochondrial 16S gene was amplified using primers 16Sar and 16Sbr (Kojima et al., 1995). A 689 bp fragment of the mitochondrial gene COI was amplified using the primers HCO and LCO (Folmer et al., 1994). Amplification was performed under the following PCR conditions: 94°C (1 min); 50°C (2 min); and 72°C (2.5 min) for 30 cycles. All PCR reactions were performed using 0.5 µl of each primer, 2.5 µl of 10XBuffer, 2 µl of 10 µM dNTPs, 0.2 µl of taq, 16.5 µl of water, and 3 µl of template. The PCR product was first purified with the ExoSap-it protocol (USB, Affimetrix) and then run on a 2% agarose gel stained with ethidium bromide to enable us to check the quantity and quality of the product. The purified PCR product was used as a template for double-stranded sequencing that was carried out at the Pennsylvania State University Sequencing Core Facility, University Park, Pennsylvania, using ABI 3730 sequencer machines.

2.3. Phylogenetic analysis

Sequences were first assembled and edited using Geneious Pro 4.0.4 (Biomatters Ltd.), and then aligned using ClustalX (Thompson et al., 2002). All alignments were confirmed and edited visually in MacClade 4.06 OS X (Maddison and Maddison, 2000) to insure that indel variation was aligned consistently among all sequenced genes.

Phylogenetic analyses of the aligned sequences were conducted using the maximum parsimony (MP) optimality criterion and neighbor joining (Saitou and Nei, 1987) (NJ) in PAUP* version 4.0b10 for Macintosh (Wilgenbusch and Swoford, 2003), and the maximum likelihood (ML) optimality criterion in GARLI v0.951.0sX-GUI (Zwickl, 2006) and PhyML (Guindon and Gascuel, 2003). The best-fit model used in PhyML and PAUP* was assessed using the akaike information criterion as implemented in modelfit (Posada, 2003; Posada and Crandall, 1998).

The best-fit model was (HKY+I+G) for the COI dataset and (GTR+G) for the 16S dataset. Clade stability was assessed by ML bootstrap analysis (Felsenstein, 1985) in GARLI (100 bootstrap replicates) and NJ (1000 replicates) in PAUP*. The ML analyses in GARLI were performed using random starting trees and default termination conditions. Within- and between-species distances were estimated in MEGA 4 (Tamura et al., 2007).

3. Results

The complete COI dataset includes 146 sequences (Table 1) of the six Gulf of Mexico (COM) cold-seep morphospecies, the available GenBank sequences of E. southwardae, E. spicata, and assorted Lamellibrachia species from around the world. Sequences from the hydrothermal vent-dwelling genera Riftia, Oasiaia, Tevnia, and Arcovestia were used as outgroups. We restricted our analyses to

![Fig. 1. Map of new deep-water collection sites in the Gulf of Mexico.](image-url)
| Sample | Clade          | Location   | GenBank Accession # | Genes      |
|--------|---------------|------------|---------------------|------------|
| 1.AC818 | *Escarbia laminata* | GOM AC818 16S: GU068165 | 16S/COI | COI: GU059163 |
| 2.AC818 | *Escarbia laminata* | GOM AC818 16S: GU068166 | 16S/COI | COI: GU059205 |
| 3.AC818 | *Escarbia laminata* | GOM AC818 16S: GU068167 | 16S/COI | COI: GU059214 |
| 4.AC818 | *Escarbia laminata* | GOM AC818 16S: GU068168 | 16S/COI | COI: GU059222 |
| 5.AC818 | *Escarbia laminata* | GOM AC818 16S: GU068169 | 16S/COI | COI: GU059228 |
| 6.AC818 | *Escarbia laminata* | GOM AC818 16S: GU068170 | 16S/COI | COI: GU059234 |
| 8.AC818 | *Lamellibrachia hyymesi* | GOM AC818 16S: GU068171 | 16S/COI | COI: GU059164 |
| 10.GB697 | *Escarbia laminata* | GOM GB697 16S: GU068172 | 16S/COI | COI: GU059170 |
| 11.GB829 | *Escarbia laminata* | GOM GB829 16S: GU068173 | 16S/COI | COI: GU059174 |
| 12.GB829 | *Escarbia laminata* | GOM GB829 16S: GU068174 | 16S/COI | COI: GU059177 |
| 13.GC600 | *Escarbia laminata* | GOM GC600 16S: GU068175 | 16S/COI | COI: GU059185 |
| 14.GC852 | *Escarbia laminata* | GOM GC852 16S: GU068176 | 16S/COI | COI: GU059192 |
| 17.GC852 | *Escarbia laminata* | GOM GC852 16S: GU068177 | 16S/COI | COI: GU059193 |
| 18.GC852 | *Escarbia laminata* | GOM GC852 16S: GU068178 | 16S/COI | COI: GU059194 |
| 19.GC852 | *Escarbia laminata* | GOM GC852 16S: GU068179 | 16S/COI | COI: GU059195 |
| 19B.AC818 | *Escarbia laminata* | GOM AC 818 16S: GU068180 | 16S/COI | COI: GU059196 |
| 20.WR269 | *Escarbia laminata* | GOM WR269 16S: GU068181 | 16S/COI | COI: GU059197 |
| 21.WR269 | *Escarbia laminata* | GOM WR269 16S: GU068182 | 16S/COI | COI: GU059198 |
| 22.WR269 | *Escarbia laminata* | GOM WR269 16S: GU068183 | 16S/COI | COI: GU059199 |
| 23.WR269 | *Escarbia laminata* | GOM WR269 16S: GU068184 | 16S/COI | COI: GU059200 |
| 24.WR269 | *Escarbia laminata* | GOM WR269 16S: GU068185 | 16S/COI | COI: GU059201 |
| 26.AT340 | *Escarbia laminata* | GOM AT340 16S: GU068186 | 16S/COI | COI: GU059202 |
| 27.AT340 | *Escarbia laminata* | GOM AT340 16S: GU068187 | 16S/COI | COI: GU059203 |
| 28.AT340 | *Escarbia laminata* | GOM AT340 16S: GU068188 | 16S/COI | COI: GU059204 |
| 29.AT340 | *Escarbia laminata* | GOM AT340 16S: GU068189 | 16S/COI | COI: GU059205 |
| 30.AT340 | *Escarbia laminata* | GOM AT340 16S: GU068190 | 16S/COI | COI: GU059206 |
| 31.AT340 | *Escarbia laminata* | GOM AT340 16S: GU068191 | 16S/COI | COI: GU059207 |
| 32.AT340 | *Escarbia laminata* | GOM AT340 16S: GU068192 | 16S/COI | COI: GU059208 |
| 33.AT340 | *Escarbia laminata* | GOM AT340 16S: GU068193 | 16S/COI | COI: GU059209 |
| 34.WR264 | *Escarbia laminata* | GOM WR264 16S: GU068194 | 16S/COI | COI: GU059210 |
| 35.WR269 | *Escarbia laminata* | GOM WR269 16S: GU068195 | 16S/COI | COI: GU059211 |
| 37.AC601 | *Escarbia laminata* | GOM AC601 16S: GU068196 | 16S/COI | COI: GU059212 |
| 38.AC601 | *Escarbia laminata* | GOM AC601 16S: GU068197 | 16S/COI | COI: GU059213 |
| 39.AC601 | *Escarbia laminata* | GOM AC601 16S: GU068198 | 16S/COI | COI: GU059214 |
| 40.AC601 | *Escarbia laminata* | GOM AC601 16S: GU068199 | 16S/COI | COI: GU059215 |
| 41.AC601 | *Escarbia laminata* | GOM AC601 16S: GU068200 | 16S/COI | COI: GU059216 |
| 42.AC601 | *Escarbia laminata* | GOM AC601 16S: GU068201 | 16S/COI | COI: GU059217 |
| 43.AC601 | *Escarbia laminata* | GOM AC601 16S: GU068202 | 16S/COI | COI: GU059218 |
| 44.AC601 | *Escarbia laminata* | GOM AC601 16S: GU068203 | 16S/COI | COI: GU059219 |
| 45.AC601 | *Escarbia laminata* | GOM AC601 16S: GU068204 | 16S/COI | COI: GU059220 |
| Sample   | Clade                        | Location | GenBank Accession # | Genes |
|----------|-----------------------------|----------|---------------------|-------|
| 48.AC601 | Escarpia laminata           | GOM AC601| GU068203            | 16S   |
| 49.AC601 | Escarpia laminata           | GOM AC601| GU068204            | 16S/COI|
| 50.AC601 | Lamellibrachia luymesi/sp. 1| GOM GC185| GU068205            | 16S   |
| 51.AT340 | Escarpia laminata           | GOM AT340| GU068206            | 16S   |
| 52.AT340 | Escarpia laminata           | GOM AT340| GU068207            | 16S   |
| 54.AC601 | Escarpia laminata           | GOM AC601| GU068208            | 16S/COI|
| 55.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068209          | 16S/COI|
| 56.L. sp. 1 GB697 | Lamellibrachia luymesi/sp. 1 | GOM GB697 | GU068210          | 16S   |
| 57.L. luymesi GC234 | Lamellibrachia luymesi/sp. 1 | GOM GC234 | GU068211          | 16S/COI|
| 58.L. sp. 1 GC852 | Lamellibrachia luymesi/sp. 1 | GOM GC852 | GU068212          | 16S/COI|
| 59.L. sp. 1 AC601 | Lamellibrachia luymesi/sp. 1 | GOM AC601 | GU068213          | 16S   |
| 60.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068214          | 16S/COI|
| 61.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068215          | 16S   |
| 62.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068216          | 16S/COI|
| 63.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068217          | 16S   |
| 64.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068218          | 16S/COI|
| 65.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068219          | 16S   |
| 66.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068220          | 16S/COI|
| 67.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068221          | 16S   |
| 68.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068222          | 16S/COI|
| 69.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068223          | 16S/COI|
| 70.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068224          | 16S/COI|
| 71.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068225          | 16S   |
| 72.L. luymesi BP | Lamellibrachia luymesi/sp. 1 | GOM GC233 | GU068226          | 16S/COI|
| 73.L. sp. 1 GB697 | Lamellibrachia luymesi/sp. 1 | GOM GB697 | GU068227          | 16S/COI|
| 74.L. sp. 1 GB697 | Lamellibrachia luymesi/sp. 1 | GOM GB697 | GU068228          | 16S   |
| 75.L. sp. 1 GB697 | Lamellibrachia luymesi/sp. 1 | GOM GB697 | GU068229          | 16S/COI|
| 76.L. sp. 1 GB829 | Lamellibrachia luymesi/sp. 1 | GOM GB829 | GU068230          | 16S/COI|
| 77.L. sp. 1 GB829 | Lamellibrachia luymesi/sp. 1 | GOM GB829 | GU068231          | 16S   |
| 78.L. sp. 1 GB829 | Lamellibrachia luymesi/sp. 1 | GOM GB829 | GU068232          | 16S/COI|
| 79.L. sp. 1 GB829 | Lamellibrachia luymesi/sp. 1 | GOM GB829 | GU068233          | 16S   |
| 80.L. sp. 1 GB829 | Lamellibrachia luymesi/sp. 1 | GOM GB829 | GU068234          | 16S/COI|
| 81.L. luymesi GC234 | Lamellibrachia luymesi/sp. 1 | GOM GC234 | GU068235          | 16S/COI|
| 82.L. luymesi GC234 | Lamellibrachia luymesi/sp. 1 | GOM GC234 | GU068236          | 16S   |
| 84.L. luymesi GC234 | Lamellibrachia luymesi/sp. 1 | GOM GC234 | GU068237          | 16S/COI|
| 85.L. luymesi GC234 | Lamellibrachia luymesi/sp. 1 | GOM GC234 | GU068238          | 16S/COI|
| 86.L. luymesi GC234 | Lamellibrachia luymesi/sp. 1 | GOM GC234 | GU068239          | 16S/COI|
| 88.L. sp. 1 GC600 | Lamellibrachia luymesi/sp. 1 | GOM GC600 | GU068240          | 16S/COI|
| 89.L. sp. 1 GC600 | Lamellibrachia luymesi/sp. 1 | GOM GC600 | GU068241          | 16S/COI|
| 90.L. sp. 1 GC852 | Lamellibrachia luymesi/sp. 1 | GOM GC852 | GU068242          | 16S/COI|
| 91.L. sp. 1 GC852 | Lamellibrachia luymesi/sp. 1 | GOM GC852 | GU068243          | 16S   |
| 92.L. sp. 1 GC852 | Lamellibrachia luymesi/sp. 1 | GOM GC852 | GU068244          | 16S/COI|
| 93.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068245          | 16S/COI|
| 94.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068246          | 16S   |
| 95.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068247          | 16S/COI|
| 96.L. luymesi BH | Lamellibrachia luymesi/sp. 1 | GOM GC185 | GU068248          | 16S   |
| 97.L. luymesi GC234 | Lamellibrachia luymesi/sp. 1 | GOM GC234 | GU068249          | 16S/COI|
| 98.L. luymesi GC234 | Lamellibrachia luymesi/sp. 1 | GOM GC234 | GU068250          | 16S/COI|
| 99.L. luymesi GC234 | Lamellibrachia luymesi/sp. 1 | GOM GC234 | GU068251          | 16S/COI|
| 100.L. sp. 1 WR269 | Lamellibrachia luymesi/sp. 1 | GOM WR269 | GU068252          | 16S   |
| 102.L. sp. 1 WR269 | Lamellibrachia luymesi/sp. 1 | GOM WR269 | GU068253          | 16S/COI|
| Sample | Clade                          | Location | GenBank Accession # | Genes |
|--------|-------------------------------|----------|--------------------|-------|
| 103.L. sp. 1 AT340 | Lamellibrachia luymesi/sp. 1 | GOM AT340 | 16S: GU868254 | 16S/COI |
| 104.L. sp. 1 WR269 | Lamellibrachia luymesi/sp. 1 | GOM WR269 | 16S: GU868255 | 16S/COI |
| 105.L. sp. 1 WR269 | Lamellibrachia luymesi/sp. 1 | GOM WR269 | 16S: GU868256 | 16S/COI |
| 107.L. sp. 1 AC601 | Lamellibrachia luymesi/sp. 1 | GOM AC601 | 16S: GU868257 | 16S/COI |
| 110.L. sp. 1 AC601 | Lamellibrachia luymesi/sp. 1 | GOM AC601 | 16S: GU868258 | 16S/COI |
| 112.GB697 | Lamellibrachia sp. 2 | GOM GB697 | 16S: GU868259 | 16S |
| 114.GB697 | Lamellibrachia sp. 2 | GOM GB697 | 16S: GU868260 | 16S/COI |
| 116.GB829 | Lamellibrachia sp. 2 | GOM GB829 | 16S: GU868261 | 16S |
| 117.GC600 | Lamellibrachia sp. 2 | GOM GC600 | 16S: GU868262 | 16S |
| 118.GC852 | Lamellibrachia sp. 2 | GOM GC852 | 16S: GU868263 | 16S |
| 119.GC852 | Lamellibrachia sp. 2 | GOM GC852 | 16S: GU868264 | 16S/COI |
| 121.GR269 | Lamellibrachia sp. 2 | GOM GR269 | 16S: GU868265 | 16S/COI |
| 122.GR269 | Lamellibrachia sp. 2 | GOM GR269 | 16S: GU868266 | 16S |
| 123.GR269 | Lamellibrachia sp. 2 | GOM GR269 | 16S: GU868267 | 16S |
| 124.GR269 | Lamellibrachia sp. 2 | GOM GR269 | 16S: GU868268 | 16S |
| 126.GC601 | Lamellibrachia sp. 2 | GOM GC601 | 16S: GU868269 | 16S/COI |
| 128.GC601 | Lamellibrachia sp. 2 | GOM GC601 | 16S: GU868270 | 16S/COI |
| 130.GB697 | Seepiophila jonesi | GOM GB697 | 16S: GU868271 | 16S/COI |
| 131.GB647 | Seepiophila jonesi | GOM GB647 | 16S: GU868272 | 16S/COI |
| 132.GC234 | Seepiophila jonesi | GOM GC234 | 16S: GU868273 | 16S/COI |
| 133.GC234 | Seepiophila jonesi | GOM GC234 | 16S: GU868274 | 16S/COI |
| 134.GC234 | Seepiophila jonesi | GOM GC234 | 16S: GU868275 | 16S/COI |
| 134b.GC234 | Seepiophila jonesi | GOM GC234 | 16S: GU868276 | 16S/COI |
| 135.GC234 | Seepiophila jonesi | GOM GC234 | 16S: GU868277 | 16S/COI |
| 136.GC234 | Seepiophila jonesi | GOM GC234 | 16S: GU868278 | 16S |
| 137.BH | Seepiophila jonesi | GOM GC234 | 16S: GU868279 | 16S |
| 138.BH | Seepiophila jonesi | GOM GC234 | 16S: GU868280 | 16S |
| 139.BH | Seepiophila jonesi | GOM GC234 | 16S: GU868281 | 16S |
| 140.GC234 | Seepiophila jonesi | GOM GC234 | 16S: GU868282 | 16S |
| 141.GB647 | Seepiophila jonesi | GOM GB647 | 16S: GU868283 | 16S/COI |
| 142.GB647 | Seepiophila jonesi | GOM GB647 | 16S: GU868284 | 16S/COI |
| 143.GB647 | Seepiophila jonesi | GOM GB647 | 16S: GU868285 | 16S/COI |
| 144.GB647 | Seepiophila jonesi | GOM GB647 | 16S: GU868286 | 16S/COI |
| 145.GC234 | Seepiophila jonesi | GOM GB647 | 16S: GU868287 | 16S/COI |
| 146.GC234 | Seepiophila jonesi | GOM GB647 | 16S: GU868288 | 16S/COI |
| 147.GC234 | Seepiophila jonesi | GOM GB647 | 16S: GU868289 | 16S |
| 148.GC234 | Seepiophila jonesi | GOM GB647 | 16S: GU868290 | 16S |
| 149.GC234 | Seepiophila jonesi | GOM GB647 | 16S: GU868291 | 16S |
| 150.GC234 | Seepiophila jonesi | GOM GB647 | 16S: GU868292 | 16S |
| 151.GC234 | Seepiophila jonesi | GOM GB647 | 16S: GU868293 | 16S |
| 152.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868294 | 16S/COI |
| 153.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868295 | 16S/COI |
| 154.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868296 | 16S/COI |
| 155.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868297 | 16S/COI |
| 156.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868298 | 16S/COI |
| 157.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868299 | 16S/COI |
| 158.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868300 | 16S/COI |
| 160.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868301 | 16S/COI |
| 161.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868302 | 16S/COI |
| 162.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868303 | 16S/COI |
| 165.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868304 | 16S/COI |
| 166.GC234 | Lamellibrachia luymesi/sp. 1 | GOM GB647 | 16S: GU868305 | 16S/COI |
| S. jonesi BH | Seepiophila jonesi | GOM GC185 | 16S: GU868306 | 16S/COI |
| S. jonesi GB425 | Seepiophila jonesi | GOM GC185 | 16S: GU868307 | 16S/COI |
| Sample† | Clade | Location§ | GenBank Accession # | Genes |
|---------|-------|-----------|---------------------|-------|
| Lamellibrachia luymesi | Lamellibrachia luymesi | GOM GC234 | AY129136 | COI |
| Besibranchia mariana | Besibranchia mariana | West Pacific | U74078 | COI |
| Arcovestia | Arcovestia ianovii | West Pacific | AB073491 | COI |
| E. laminata | Escarpia laminata | West Atlantic | U74063 | COI |
| E. southwardae | Escarpia southwardae | West Africa | AY362304 | COI |
| E. southwardae | Escarpia southwardae | West Africa | AY362303 | COI |
| E. spicata | Escarpia spicata | East Pacific | U54262 | COI |
| L. sp.1_b | Lamellibrachia luymesi/sp. 1 | GOM AT340 | U74061 | COI |
| Oasisia HaploA | Oasisia alvinae | East Pacific | AY646001 | COI |
| Lam.2000Nanaki | Lamellibrachia sp. | West Pacific | D30592 | COI |
| Lam.3000Sagami | Lamellibrachia sp. | West Pacific | AY886774 | COI |
| Lam.3000Sagami 1 | Lamellibrachia sp. | West Pacific | D38029 | COI |
| Lam bara10b | Lamellibrachia barhami | East Pacific | AY129137 | COI |
| Lam bara11b | Lamellibrachia barhami | East Pacific | AY129138 | COI |
| Lam bara1b | Lamellibrachia barhami | East Pacific | AY129147 | COI |
| Lam bara1 | Lamellibrachia barhami | East Pacific | AY129146 | COI |
| Lam bara1b | Lamellibrachia barhami | East Pacific | AY129145 | COI |
| L. barhami2 | Lamellibrachia barhami | East Pacific | AF315045 | 16S |
| L. barhami3 | Lamellibrachia barhami | East Pacific | AF315045 | 16S |
| Lam columnna | Lamellibrachia columna | West Pacific | U74061 | COI |
| Lam columna | Lamellibrachia columna | West Pacific | AB055210 | COI |
| Lam juni | Lamellibrachia junci | West Pacific | AB242858 | COI |
| Lam juniHaplo1 | Lamellibrachia junci | West Pacific | AB264601 | COI |
| Lam juniHaplo2 | Lamellibrachia junci | West Pacific | AB264602 | COI |
| Lam juniHaplo3 | Lamellibrachia junci | West Pacific | AB264603 | COI |
| Lam juniHaplo4 | Lamellibrachia junci | West Pacific | AB264604 | COI |
| Lam juniHaplo5 | Lamellibrachia junci | West Pacific | AB264605 | COI |
| Lam L4 | Lamellibrachia sp. | West Pacific | AB055209 | COI |
| Lam 5 | Lamellibrachia sp. | West Pacific | AB055210 | COI |
| Lam 6 | Lamellibrachia sp. | West Pacific | AB088674 | COI |
| Lam 7 | Lamellibrachia sp. | West Pacific | AB088675 | COI |
| LamaymesiBH 2 | Lamellibrachia luymesi | GOM GC185 | AY129133 | COI |
| LamaymesiBHb | Lamellibrachia luymesi | GOM GC185 | AY129132 | COI |
| LamaymesiBHc | Lamellibrachia luymesi | GOM GC185 | AY129139 | COI |
| LamaymesiGB4252 | Lamellibrachia luymesi | GOM GB425 | AY129135 | COI |
| LamaymesiGC354 | Lamellibrachia luymesi | GOM GC354 | AY129126 | COI |
| Lamaymesi VK | Lamellibrachia luymesi | GOM VK26 | AY129124 | COI |
| Lam Med | Lamellibrachia sp. from Med. | Mediterranean | EU046616 | COI |
| Lam satsumab | Lamellibrachia satsumensis | West Pacific | AF342671 | COI |
| NewEscarpiidGB425 | Escarpid sp. nov. | GOM GB425 | AY129134 | COI |
| Oasisia fujikurai | Oasisia fujikurai | South/West Pacific | AB242857 | COI |
| Paraescarpia | Paraescarpia cf. echinopsina | West Pacific | D50594 | COI |
| Ridgea | Ridgea piscacea | Juan de Fuca Ridge | AF315054 | 16S |
| Ridgea | Ridgea piscacea | Juan de Fuca Ridge | AF315054 | 16S |
| Ridgea | Ridgea piscacea | Juan de Fuca Ridge | AF315054 | 16S |
| Ridgea | Ridgea piscacea | Juan de Fuca Ridge | AF315054 | 16S |
| Rifina | Rifina pachyphtyla | East Pacific | AY459899 | COI |
| Tevnia jerichonana | Tevnia jerichonana | East Pacific | 16S: AF315042 | COI |
| S. jonesi BH | Seepiophila jonesi | GOM GC185 | AY317287 | COI |
| S. jonesi GB425 | Seepiophila jonesi | GOM GB425 | AY317288 | COI |

† Samples analyzed for this study are numbered and labeled as for Figs. 2 and 3. Sequences from Genbank are listed by names assigned in Genbank.
§ Samples from the Gulf of Mexico are indicated by GOM followed by the abbreviation of their collection sites. V1K26, GC185, GC233, GB425, GC234, and GC354 are all on the upper Louisiana slope at depths < 800 m. The other GOM sites are at depths > 900 m and are indicated on Fig. 1.

the species’ boundaries for Lamellibrachia, Escarpia, and Seepiophila, and we do not infer higher level phylogenetic relationships among genera because neither 16S nor COI offers sufficient resolution at deeper nodes. The complete and aligned COI dataset included 690 bp, of which 460 were invariant sites, 207 were phylogenetically informative sites, and 23 were autapomorphies. The complete 16S dataset consisted of 133 sequences (see Table 1 for the complete list of samples), 127 of which were from the Gulf of Mexico. Sequences from the vent-dwelling genera Tevnia and Ridgea were used as outgroups. The aligned 16S dataset consisted of 524 bp, of which 433 were invariant sites, 72 were phylogenetically informative, and 19 were autapomorphies. MP, ML, and NJ analyses produced congruent trees, and the GARLI ML phylogeny is presented in Fig. 2 A and B and 3 A and B. Both 16S and COI phylogenies identify five distinct monophyletic clades of vestimentiferans in the Gulf of Mexico. Four of the clades represent single morphospecies, S. jonesi, E. laminata, Lamellibrachia sp. 2, and escarpiid sp. nov., from the upper slope. However, the fifth clade includes both Lamellibrachia sp. 1 from the collections in the deeper GOM and L. luymesi from the upper Louisiana slope sites. They were, therefore, considered a single species when within- and between-species distances for the 16S and COI datasets were estimated. Additionally, COI sequences of E. laminata did not differ from those of E. spicata and
E. southwardae from the East Pacific and East Atlantic, respectively. We were unable to obtain 16S sequences for E. spicata or E. southwardae.

Estimates of within- and between-species diversity (p) for both genes are shown in Tables 2 and 3. Within a species, p distances range from 0% to 0.1% for 16S and 0% to 0.9% for the more variable COI. The very low values for the undescribed escarpiid may reflect the small number of individuals of this species analyzed (n = 3 for COI and n = 2 for 16S).

4. Discussion

4.1. Distribution of vestimentiferan species in the Gulf of Mexico and relation to other seep species

Vestimentiferans have been collected from both hydrothermal vent and cold-seep sites. The vent and seep species fall into two different clades. However, it should be noted that “seep species” are sometimes found in sedimented hydrothermal vent areas with low levels of diffuse flow, and that “cold-seep” fluids may have temperatures elevated over background (Black et al., 1998; Kojima et al., 1997; MacDonald et al., 2000; Joye et al., 2005); so this separation really reflects more aspects of their habitat than temperature alone. Vestimentiferans found at cold seeps worldwide can be further divided into two clades. One clade includes at least five named and three unnamed species in the genus Lamellibrachia. The other clade includes three named species in the genus Escarpia, S. jonesi, Paraescarpia echinospica, and a rarely collected species (escarpiid sp. nov.) from the shallow GOM. Although Arcovestia seems basal to the Lamellibrachia clade (Fig. 2B), this position is not well supported.

Three species in the escarpiid clade of seep vestimentiferans are found in the GOM: S. jonesi has been collected from numerous sites,
ranging in depth from 500 to 950 m; escariiid sp. nov. from two sites ranging in depth from 600 to 640 m, where it co-occurs with S. jonesi (although it has been reported also from GC234 at 525 m; see Cordes et al., 2003); and E. laminata from 950 to 3200 m depth. S. jonesi and E. laminata co-occurred at only one site, GB847, at a depth of 950 m. The undescribed escariiid differs morphologically from S. jonesi, as it lacks the curl of the ventral vestimental fold that is a defining character of the genus Seepiophila (Gardiner et al., 2001). Additionally, the obturacular process of the undescribed escariiid forms a spike, whereas it is flat in S. jonesi and barely protrudes from the top of the obturaculum.

Both the COI and 16S phylogenetic trees distinguish these three species and place them within the escariiid clade of seep vestimentiferans (Figs. 2 and 3). Both the 16S tree and the 16S p distance matrix suggest E. laminata is more closely related to S. jonesi (between-species uncorrected p = 2%) than to the undescribed escariiid (between-species uncorrected p = 3.5%). However, the COI tree groups the undescribed escariiid with the described Escarpia spp. The bootstrap value based on COI data supporting this clade is low (61%), and the grouping observed for the 16S dataset has a bootstrap below 50%. Neither tree allows us to state clearly whether this new escariiid is more closely related to Escarpia, Paraescarpia, or Seepiophila.

As previously noted by other authors, COI does not separate Escarpia southwardae, E. spicata, and E. laminata, respectively, from cold seeps on the west coast of Africa in the eastern Atlantic, Guaymas Basin, off the coast of Mexico, and the GOM (Black et al., 1998). Also, there is very little to no intra-clade diversity within this group (Table 3). This result may indicate that those three nominal species represent a single genealogical species with a surprisingly wide geographic distribution and variable morphology. However, this assumption would require a high level of gene flow between quite distant localities, especially since the closing of the Isthmus of Panama 3.5 million years ago, followed the closing of the deep sea exchange 10 million years ago (Burton et al., 1997). This level of genetic exchange over these distances seems quite unlikely, considering what is known about larval development times for vestimentiferans (Marsh et al., 2001, Young et al., 1996). Although the life span of Escarpia larvae has not been determined, the larval life span of the vent species Riftia pachyptila is estimated at about three weeks (Marsh et al., 2001) and the larval life span of the seep vestimentiferan L. laymesi is estimated to be about one month (Young et al., 1996). Tyler and Young (1999) estimate that the maximal dispersal distances for these species are on the order of 60 km per generation, which is unlikely to support the level of genetic mixing necessary to maintain genetic homogeneity among the three described species of Escarpia from such widely separated geographic locations. It is possible, however, that undiscovered seeps around South America could connect all of these species.

The lack of fixed COI differences within Escarpia spicata, E. laminata, and E. southwardae could also be due to different rates of evolution of the COI gene in different taxa. COI has been used for higher level phylogenetic reconstructions in other groups of annelids (Halanych and Janosik, 2006) and has been adopted as an appropriate gene for the “barcode of life” for animals in general by the barcode of life initiative (BOLI; http://www.dnabarcodes.org/). However, the fact that COI fails to identify morphologically distinct populations of Escarpia from such widely separated areas implies that in this clade the mutation rate may be considerably slower than in other lineages. Slower rates of evolution in the mitochondrial DNA have been recognized in some other groups, such as the cnidarian class Anthozoa, where this phenomenon has been linked to an especially efficient repair system of their mitochondrial DNA (France and Hoover, 2002; Pont-Kingdon et al., 1998); however, no evidence of a similar system has been found in vestimentiferan mitochondrial DNA. Seep vestimentiferans can also be extremely long-lived (Bergquist et al., 2000; Cordes et al., 2001a), which may contribute to a slower rate of change of mitochondrial DNA (see for example Nabholz et al., 2008) for a consideration of longevity effects on mitochondrial rates of evolution in vertebrates.

In the COI dataset, the Lamellibrachia clade is divided into eight distinct groups that represent presumptive species, including five basal species (L. juni, L. barhami, L. satsuma, L. sp. Japan, and L. sp. West Pacific), all of which are from the Pacific Ocean and four of which are from the western Pacific. This observation is consistent with the hypothesis that the genus Lamellibrachia originated in the Pacific, likely the western Pacific, and subsequently radiated to the eastern Pacific, the Atlantic, and the GOM. Three morphological species of Lamellibrachia were identified in collections from the GOM: L. laymesi, from the upper slope at
between about 400 m and 800 m; L. sp. 1, from 950 to 2320 m; and L. sp. 2, from 1175 to 2320 m. L. luymesi and L. sp. 1 have a similar number of sheath lamellae, but the deep-water L. sp. 1 generally has more gill lamellae, ranging between 21 and 27 in the 28 individuals examined, whereas the shallow-water L. luymesi has between 15 and 22 gill lamellae in the 20 individuals examined for the species description. The morphological character that allowed rapid identification of animals aboard the ship was the relatively short and fat vestimentum of L. sp. 1. The ratio of length to width of the vestimentum of L. sp. 1 ranges from 2.4 to 4.7 and from 6.2 to 16.4 in L. luymesi. L. sp. 2 has a similar number of sheath and gill lamellae as L. sp. 1, and the vestimentum length to width ratio tends to be shorter (1.9 to 3). The most distinct field character for L. sp. 2 is the lack of a ventral vestimental fold, which is present on L. sp. 1.

Despite morphological characters that distinguish the three GOM *Lamellibrachia* presumptive species, only either the COI or the 16S phylogenetic trees resolved two of them. Specifically, both genes failed to separate L. luymesi from the shallow GOM and L. sp. 1 from the deeper GOM sites. This lack of genetic differences between individuals that span such a wide depth range is unusual (Chase et al., 1998; Zardus et al., 2006) and surprising, given the morphological differences. Both 16S and COI genes consistently identify *Lamellibrachia* sp. 2 as a separate clade, sister to the L. luymesi/L. sp. 1 clade.

There were no apparent geographic distributional patterns that were independent of depth for the seep vestimentiferans in the Gulf of Mexico. The common species present on the upper Louisiana slope (L. luymesi and *S. jonesi*) have been found at both the eastern-most and western-most sites where we have collected vestimentiferans. *E. laminata* from the lower slope range from the Alaminos Canyon sites, our most westerly collection sites for this study, to the Florida Escarpment in the eastern GOM (Cordes et al., 2009; McMullin et al., 2003). Both of the *Lamellibrachia* spp. found at the deeper sites occurred over the entire E–W range of sites within their depth range (from the Alaminos Canyon sites in the west to AT340 in the east).

4.2. Within-species diversity of the GOM vestimentiferans

Tables 2 and 3 report within- and between-species *p* distance calculated for the GOM genetic species. In most cases, within-species diversity for both 16S and in the COI genes is strikingly low, a finding that is in contrast to previous studies on deep-sea mollusks and echinoderms, where large amounts of genetic variation were observed over small distances (Chase et al., 1998; Howell et al., 2004; Quattro et al., 2001). However, large-scale studies indicate that low within-species genetic variation may be typical of deep-sea organisms (Bisol et al., 1984) and even suggest that it may decrease with increase in depth (France and Kocher, 1996). Genetic variation has been suggested to be an important feature of the genome of an organism that allows it to adapt to a changing environment (Powers et al., 1991). Organisms that live in the deep sea may experience a long-term stable environment, resulting in low levels of within-species genetic diversity. Alternatively, low within-species genetic diversity may be the result of fewer replication errors, more efficient repair in the germ line, or repeated population bottlenecks.

*E. laminata*, *E. spicata*, and *E. southwardae clade* and *L. luymesi* sp. 1 and *L. sp. 2* have a moderate degree of intra-specific diversity (Figs. 2 and 3). However, as with all of the GOM vestimentiferans analyzed, none of the within-species clades grouped by specific geographic locations or depth. A similar pattern was found in the seep mussel *Bathymodiolus childressi*, which, based on markers ranging from microsatellites to mitochondrial genes, has a panmictic population in the GOM ranging across 550 km east to west and from 540 to 2200 m depth (Carney et al., 2006; Cordes et al., 2007b). In contrast, genetic breaks and barriers that restrict gene flow were identified in both hydrothermal vent vestimentiferans and mussels along the East Pacific Rise (EPR). Specifically, Won et al. (2003) used COI sequences to identify two highly divergent clades on the EPR on the two sides of the Easter Island Microplate. Similarly, Hurtado et al. (2004) used COI sequences to identify several geographic breaks and barriers that restrict gene flow in three genera of annelids along the EPR, including two species of vestimentiferan (*Riftia pachyptila* and *Tevnia jerichonana*).

5. Summary

In this study, our primary goals were to identify and characterize the distributions of vestimentiferans at seep sites covering a wide geographic and depth range in the Gulf of Mexico and to investigate their relationship to other seep vestimentiferan species, using phylogenetic analysis of mitochondrial gene sequences. Although the genetic analyses confirmed identification of most of the morphological species during collections, we also identified an unexpected discrepancy between the morphospecies identified during the collections and genealogical species identified using the mitochondrial genes COI and 16S. Using morphological characters, we identified two new species of *Lamellibrachia* (sp. 1 and 2). However, neither COI nor 16S distinguished the deeper occurring morphospecies *L. sp. 1* from *L. luymesi*, the common *Lamellibrachia* species on the upper Louisiana slope. Our molecular genetic analyses confirm the presence of three vestimentiferan species within the escarpid clade in the Gulf of Mexico. However, since COI also does not differentiate between *E. laminata* found in the Gulf of Mexico and the other described *Escarpia* species off the coast of Africa or in the eastern Pacific Ocean, we suggest that COI or 16S genes may not reliably distinguish closely related species of long-lived seep vestimentiferans. We are currently evaluating the usefulness of several nuclear genes to clarify the relationships among the named species of *Escarpia* and the *Lamellibrachia* species in the Gulf of Mexico.

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