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ORIGINAL PAPER

# Island endemism, morphological stasis, and possible cryptic speciation in two coral reef, commensal Leucothoid amphipod species throughout Florida and the Caribbean

**Vincent P. Richards · Michael J. Stanhope · Mahmood S. Shivji**

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**Abstract** Coral reefs are believed to be one of the most diverse ecosystems, but the true magnitude of their biodiversity and patterns of endemism is uncertain. This uncertainty stems partly from the relative paucity of investigations on the small, difficult to collect taxa (cryptofauna) that may make up the majority of reef biodiversity and require specialized expertise for morphological identification. To assess the extent of diversity in some of the reef micro-cryptofauna, we analyzed 414 bp of the mitochondrial cytochrome oxidase subunit I gene from 556 individuals representing two brooding amphipod species (*Leucothoe ashleyae* and *Leucothoe kensleyi*). These amphipods are commensal inside the branching vase sponge *Callyspongia vaginalis*, and were sampled throughout Florida and the Caribbean. Phylogenetic analyses revealed 11 deeply divergent, strongly supported lineages (seven *L. ashleyae* and four *L. kensleyi*) each with very narrow geographic ranges. The level of intraspecific lineage divergence for both morphospecies was among the highest reported for any marine crustacean (12.4–26.0% uncorrected), and exceeded that of congeners from nine diverse amphipod families, as well as the patristic genetic distance suggested as a threshold for crustacean species delineation. These findings suggest a history of cryptic speciation within each morphospecies, concomitant with a pronounced period of morphological stasis involving each of the morphotypes. The observation of multiple, highly divergent, evolutionary significant units, each endemic to Florida and Caribbean island locations, supports the emerging view that coral reef biodiversity, especially in the cryptofaunal component, is likely vastly underestimated.

V. P. Richards · M. S. Shivji (✉)
Oceanographic Center, National Coral Reef Institute, Nova Southeastern University,
8000 North Ocean Drive, Dania Beach, FL 33004, USA
e-mail: mahmood@nova.edu

*Present Address:*
V. P. Richards
Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine,
Cornell University, Ithaca, NY 14853, USA

M. J. Stanhope
Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine,
Cornell University, Ithaca, NY 14853, USA

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## Introduction

Globally, coral reefs are severely threatened and showing rapid declines in the biodiversity of the typically studied, larger reef species such as fishes and corals (Roberts et al. 2002; Hughes et al. 2003; Knowlton and Jackson 2008). However, the majority of reef biodiversity is likely made up of smaller, lesser-studied cryptofauna, and the impacts of reef degradation on this important ecological component are unknown (Plaisance et al. 2009). Part of the problem in assessing these impacts is that biodiversity estimates of coral reefs, and especially the micro cryptofauna, are highly uncertain (Plaisance et al. 2009).

The ability to accurately describe and quantify reef biodiversity has historically been restricted by traditional taxonomic methods (Knowlton 2000). For example, there is increasing evidence to show that many marine species are complexes of multiple genetically divergent lineages, which are regarded by many as either separate "phylogenetic" species or distinct evolutionary significant units (ESUs) (Moritz 1994; De Queiroz 1998; Avise 2000; Knowlton 2000; Hellberg 2009). In addition, these components often have much narrower geographic ranges than the original morphological species (Lee 2000; Lee and Ó Foighil 2004; Meyer et al. 2005), likely rendering them more vulnerable to environmental impact and local extinction (Roberts and Hawkins 1999). To confound the issue further, genetic divergence is often coupled with morphological stasis (Knowlton 2000; Bickford et al. 2007), making it difficult to detect these cryptic lineages based on morphological criteria alone. This situation has provided a conservative view of biodiversity and impeded our understanding of basic ecological and evolutionary processes in the marine realm. For example, the dispersal capability of many species may have been severely overestimated, cryptic species invasions missed, and the fossil record misinterpreted (e.g. Geller et al. 1997; Garcia-Rodriguez et al. 1998; King and Hanner 1998).

Most genetic population studies within the Caribbean archipelago have focused on species with pelagic dispersal phases, with results showing a pattern of low genetic differentiation throughout the region (Mitton et al. 1989; Silberman et al. 1994; Lessios et al. 2001; Baums et al. 2005; Rocha et al. 2005). Notable exceptions to this trend include a goby, a mussel, and an acroporid coral (Taylor and Hellberg 2003; Lee and Ó Foighil 2005; Vollmer and Palumbi 2007). Although populations of these species were genetically partitioned, the divergent lineages extended across large geographic areas. Fine scale genetic differentiation and possible speciation among individual islands is expected to be more likely in those species with limited dispersal ability. Indeed, Goodbody-Gringley et al. (2010) recently showed differentiation among proximate Caribbean reefs for a brooding coral and Meyer et al. (2005) showed high levels of island endemism throughout the Indo-west Pacific for a turbinid gastropod whose pelagic larval phase is completed in days. Leucothoid amphipods provide an excellent model organism to further test this expectation within the Caribbean, as all amphipods brood their young and as such lack a pelagic larval phase. Moreover, leucothoid amphipods are commensal inhabitants of ascidians, bivalves, and sponges, and the often-patchy distribution of these hosts should further restrict the dispersal ability of the amphipod.

The amphipods *Leucothoe ashleyae* and *Leucothoe kensleyi* are both commensal inside the common branching vase sponge *Callyspongia vaginalis* and are distributed throughout Florida and Caribbean reefs. A previous study on *L. ashleyae* showed a high degree of

genetic partitioning, including a K2P genetic distance of 20.3% in the mitochondrial cytochrome *c* oxidase subunit one gene (hereafter COI), between the reefs of south east Florida and Glovers Reef atoll in Belize (Richards et al. 2007); this distance was comparable to the genetic distance used to infer the presence of cryptic species in other amphipod species (e.g. Witt and Hebert 2000; Witt et al. 2006). These initial findings in the context of little available information on biodiversity of Caribbean reef cryptofauna and increasing cases of cryptic species discovery in the oceans (Hellberg 2009) prompted us to investigate the genetic diversity in these amphipods and test the hypothesis that the morphospecies *L. ashleyae* and *L. kensleyi* are composed of cryptic species complexes. We conducted this assessment of diversity by utilizing sequence data from the mitochondrial COI gene based on the demonstrated utility of this locus for reliable crustacean species delimitation (Lefébure et al. 2006; Costa et al. 2007; Plaisance et al. 2009; Radulovici et al. 2009).

## Materials and methods

We collected 162 *L. ashleyae* individuals from seven island locations throughout the Caribbean: Carrie Bow Cay and Pelican Cay, Belize; Roatan, Honduras; Curaçao, Netherlands Antilles; Vieques, Puerto Rico; and Bimini, Bahamas (Fig. 1). In Roatan we collected animals from two sites on the western tip of the island and also from a large patch reef 2 km offshore of the south coast. In Vieques we sampled three sites along 9 km of the south coast and in Curaçao we sampled two sites along 27 km of the west coast. We also collected 60 *L. kensleyi* individuals from the same sites in Curaçao, Vieques, and Bimini (this species could not be found in Belize and Roatan). The animals collected above were added to 334 animals previously collected from Florida to Belize (*L. ashleyae* = 136; *L. kensleyi* = 198, Richards et al. 2007) for a total of 556 individuals. Using SCUBA, amphipods were collected from individual sponges in a variety of reef environments down to a maximum depth of 23 m. During the course of our collections, we observed an often-patchy distribution of *L. ashleyae* and *L. kensleyi* among individual *C. vaginalis* hosts, which in turn required multiple dives over a period of several days to collect sufficient individuals for analysis. The number of amphipods collected from host sponges at sampling sites throughout the Caribbean is shown in Table 1. See Richards et al. (2007) (Table 1) for details regarding Florida collection sites. All animals were identified microscopically using the keys of Thomas and Klebba (2007) with assistance of these authors where necessary, and preserved in 95% ethanol at 4°C for subsequent genetic analyses.

### Polymerase chain reaction (PCR) and sequencing

Genomic DNA was extracted from whole individual amphipods (each about the size of a rice grain) using the Dneasy Tissue Kit (Qiagen Inc.). Approximately 655 bp of the COI gene was initially amplified and sequenced using the primer pair LCO1490 and HCO2198 (Folmer et al. 1994); however, these primers did not produce optimal sequence data. Several combinations of primers (many specific to geographic sampling location) were subsequently designed to improve PCR and sequencing reaction performance (Table 2). The final primer pairs produced 414 bp of sequence data.

Total PCR volumes were 50 µl and contained 1 µl of the extracted genomic DNA, 5 µl 10× PCR Buffer, 50 µM of each dNTP, 0.25 µM of each primer, and 0.75–1.75 units of HotStar Taq™ DNA Polymerase (Qiagen Inc.). PCR was performed in a Mastercycler Gradient (Eppendorf Inc.) thermal cycler as follows: 95°C initial heating for 15 min to activate

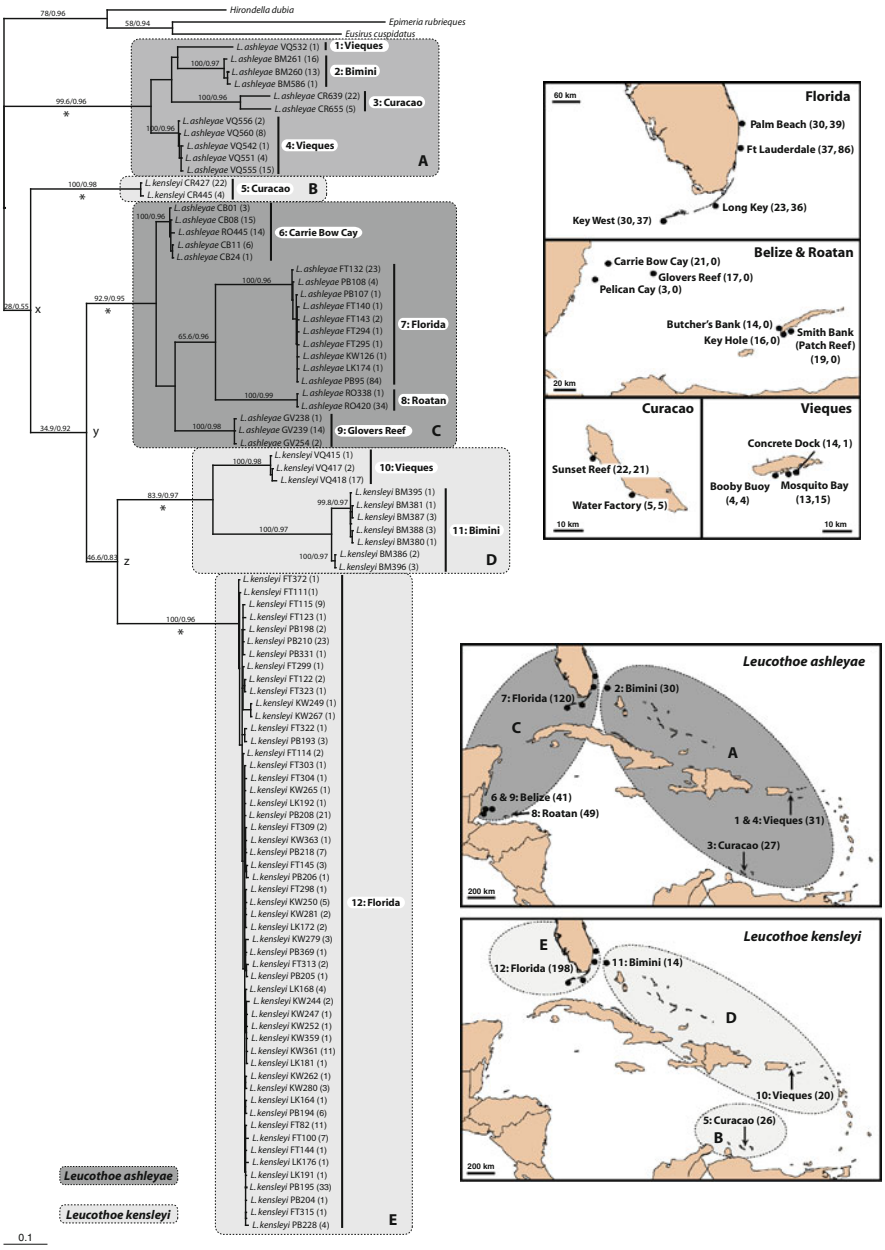


**Fig. 1** ML phylogram based on 414 bp of the mtDNA COI gene depicting the relationship among haplotypes. *Light shading* indicates *L. ashleyae* morphology and *dark shading* indicates *L. kensleyi* morphology. ML bootstrap values followed by Bayesian posterior probabilities are above branches. The number of individuals sharing a particular haplotype is indicated in parenthesis. x, y, z denote collapsed nodes in the ML bootrap consensus tree with the branches marked with an *asterisk* forming a polytomy. *Upper inset* map depicting sampling locations throughout Florida and the Caribbean. *Lower inset* map depicting individual sampling sites in Belize, Roatan, Curaçao, and Vieques. Sample sizes are shown in parenthesis *L. ashleyae* followed by *L. kensleyi*

the hot start DNA polymerase, followed by 35–45 cycles of 94°C for 1 min, 40–50°C for 1–2 min, 72°C for 1–2 min, and a 5 min final extension step at 72°C. Individual amphipods often yielded very low amounts of genomic DNA, consequently the PCR thermal profile and Taq polymerase concentration were empirically adjusted (within the above parameters) to increase amplification efficiency. As a check for reagent contamination, a negative control (no genomic DNA) was included in each PCR. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.) and sequenced in both directions on an ABI 3730xl genetic analyzer. Individual haplotype sequences are available from GenBank (accession numbers JQ004939–JQ004956 and *EF053411–EF053423 for *L. ashleyae*, and JQ004957–JQ004968 and *EF053456–EF053503 for *L. kensleyi* [*Richards et al. 2007]).

## Data analysis

GENEDOC version 2.6.02 (Nicholas et al. 1997) was used to align, edit, and translate individual COI sequences. Amino acid sequences were checked for correct invertebrate mtDNA amino acid coding and aberrant start/stop codons. Rates of synonymous and nosynonymous substitution were calculated using Nei and Gojobori's method as implemented in MEGA3 (Kumar et al. 2004). The *Z* test outlined by Nei and Kumar (2000) was used to test for significant difference between the substitution rates. We tested for substitution saturation at the third codon position using the Xia et al. (2003) method as implemented in the program DAMBE version 4.2.13 (Xia and Xie 2001). DNASP version 4.0 (Rozas et al. 2003) was used to estimate genetic diversity.

We tested for base composition bias among lineages with a $\chi^2$ test of homogeneity of base frequencies across taxa in PAUP version 4.0b10 (Swofford 2002). MODELTEST version 3.06 (Posada and Crandall 1998) selected the HKY + G + I model of molecular evolution as the best fit for the haplotype data. ML phylogenetic reconstructions were performed using PAUP. Heuristic searches obtained starting trees via stepwise addition using ten random addition sequence replicates and branch swapping was performed using tree bisection-reconnection (TBR). Additional ML phylogenetic reconstructions were performed on the translated amino acid sequences using PHYML (Guindon and Gascuel 2003) incorporating the MtREV model of mitochondrial amino acid substitution (Adachi and Hasegawa 1996). Bayesian phylogenetic reconstructions were performed using MRBAYES version 3.0b4 (Huelsenbeck and Ronquist 2001). Markov chain Monte Carlo (MCMC) sampling was initiated with a random tree and run for 2,000,000 generations. Metropolis coupling with one cold chain and three heated was used to improve MCMC sampling. The data was partitioned according to codon position and trees sampled every 100 generations. To ensure topological convergence, three replicate runs were performed.

Statistical support for branch nodes was assessed using nonparametric bootstrapping for the maximum likelihood (ML) analyses using PHYML (1,000 replicates) and posterior probabilities for Bayesian analyses (2,000,000 generations). Bootstrap values $\geq$70% and posterior probabilities $\geq$0.95 were considered well supported. Reciprocal monophyly for *L. ashleyae* and *L. kensleyi* was assessed using the SH test (Shimodaira and Hasegawa 1999) as implemented in PAUP. Due to the uncertainty of higher-level amphipod taxonomy, GenBank sequences of marine amphipods from three separate families were selected as outgroups: *Epimeria rubrieques* (Epimeriidae), *Hirondella dubia* (Lysianassidae), and *Eusirus cuspidatus* (Eusiridae).

For comparative purposes, genetic distances were calculated among all 556 individuals and also between pairs of amphipod congeners (identical region of COI) representing nine different families (Table 3). Uncorrected (*p*) and K2P corrected sequence distances were

**Table 1** Number of amphipods collected from host sponges at sampling sites throughout the Caribbean

| Sampling region and site | Sponges/site | Species | Host sponge ID | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | ? | Amphipods/site |
| Bimini | | | | | | | | | | | | | | | | | | | | | | |
| Round Rock | 18 | *L. ashleyae* | 3 | | 3 | | 2 | 2 | 3 | 2 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | 1 | 2 | 2 | | 30 |
| | | *L. kensleyi* | | 1 | 1 | 1 | | | | 1 | | 1 | | 2 | 1 | | | 2 | | 4 | | 14 |
| Vieques | | | | | | | | | | | | | | | | | | | | | | |
| Booby Bouy | 4 | *L. ashleyae* | | 2 | 1 | 1 | | | | | | | | | | | | | | | | 4 |
| | | *L. kensleyi* | 2 | | 1 | 1 | | | | | | | | | | | | | | | | 4 |
| Concrete Dock | 5 | *L. ashleyae* | 3 | 2 | 5 | 1 | 3 | | | | | | | | | | | | | | | 14 |
| | | *L. kensleyi* | | | | 1 | | | | | | | | | | | | | | | | 1 |
| Mosquito Bay | 6 | *L. ashleyae* | 4 | 3 | 1 | 1 | 1 | 3 | | | | | | | | | | | | | | 13 |
| | | *L. kensleyi* | 15 | | | | | | | | | | | | | | | | | | | 15 |
| Curacao | | | | | | | | | | | | | | | | | | | | | | |
| Sunset Reef | 10 | *L. ashleyae* | 2 | | 1 | 2 | 5 | 4 | 3 | 2 | | 3 | | | | | | | | | | 22 |
| | | *L. kensleyi* | | 5 | 4 | 4 | | 1 | 3 | 2 | 1 | | | | | | | | | | 1 | 21 |
| Water Factory | 6 | *L. ashleyae* | | | | | | | | | | | 1 | 1 | 1 | | 1 | 1 | | | | 5 |
| | | *L. kensleyi* | | | | | | | | | | | | | | 5 | | | | | | 5 |
| Roatan | | | | | | | | | | | | | | | | | | | | | | |
| Butcher's Bank | 3 | *L. ashleyae* | 5 | 2 | 5 | | | | | | | | | | | | | | | | 2 | 14 |
| Key Hole | 3 | *L. ashleyae* | 3 | 5 | 6 | | | | | | | | | | | | | | | | 2 | 16 |
| Smith Bank | 5 | *L. ashleyae* | 6 | 7 | 4 | 1 | 1 | | | | | | | | | | | | | | | 19 |
| Belize | | | | | | | | | | | | | | | | | | | | | | |
| Carie Bow Cay | 11 | *L. ashleyae* | 1 | 6 | 2 | 1 | 1 | 3 | 1 | 1 | 1 | 1 | 3 | | | | | | | | | 21 |
| Glovers Reef | 5 | *L. ashleyae* | 1 | 2 | 4 | 2 | 6 | | | | | | | | | | | | | | 2 | 17 |
| Pelican Cay | 1 | *L. ashleyae* | 3 | | | | | | | | | | | | | | | | | | | 3 |

Second column (sponges/site) shows the total number of sponges sampled at each collection site for both species of amphipod. The question mark indicates amphipods for which only collection site was known. See Richards et al. (2007) (Table 1) for details regarding Florida collection sites

**Table 2** Primer sequences

| | |
|---|---|
| *L. ashleyae* | |
| Bimini and Vieques | |
| Ls(3)COI(BIM)F1 | 5′-ATCATCCGGACAGAACTATCTTCCCC-3′ |
| Ls(3)COI(BIM)R1 | 5′-TGTGATAGCCCCAGCTAGTACAGG-3′ |
| Curaçao | |
| Ls(3)COI(CUR)R1 | 5′-AAATGTTGGTAGAGGATAGGGTCCCC-3′ |
| Carrie Bow Cay | |
| Ls(4)COIF2 | 5′-ATTATTCGAACAGAATTATCAACCCC-3′ |
| Ls(4)COIR2 | 5′-TGTAATGGCTCCCGCTAAAACTGG-3′ |
| Glovers Reef | |
| Ls(3)COI(GVS)F1 | 5′-TATTATTCGAACAGAATTATCAACCCC-3′ |
| Ls(3)COI(GVS)F2 | 5′-AACCGAATTATCAACCCCTGGAAATTTAAT-3′ |
| Ls(3)COI(GVS)R1 | 5′-TGTAATGGCTCCCGCTAAAACTGGTA-3′ |
| Roatan | |
| Ls(3)COI(FL)F1 | 5’-AACAGAATTATCCACCCCGGGAAATTTAAT-3′ |
| Ls(4)COIR2 | 5′-TGTAATGGCTCCCGCTAAAACTGG-3′ |
| Ls(3)COI(GVS)F2 | 5′-AACCGAATTATCAACCCCTGGAAATTTAAT-3′ |
| Ls(3)COI(GVS)R1 | 5′-TGTAATGGCTCCCGCTAAAACTGGTA-3′ |
| *L. kensleyi* | |
| Bimini | |
| Ls(4)COIF2 | 5′-ATTATTCGAACAGAATTATCAACCCC-3′ |
| Ls(4)COIR2 | 5′-TGTAATGGCTCCCGCTAAAACTGG-3′ |
| Veiques | |
| Ls(4)COI(VEQ)F1 | 5′-TCGAACAGAATTATCAACCCCTGGTAA-3′ |
| Ls(4)COI(VEQ)R1 | 5′-AAAACTGGTAGAGATAGTAATAGAAGAAT-3′ |
| Curaçao | |
| Ls(4)COI(CUR)R2 | 5′-CGATCTGTCAGTAGTATCGTAATAGCT-3′ |

The forward primer LCO1490 was used for both *L. ashleyae* and *L. kensleyi* collected from Curaçao

calculated using MEGA3 and GTR + G + I patristic distances were calculated from ML trees generated in PAUP using the program PATRISTICv1.0 (Fourment and Gibbs 2006). Heuristic searches obtained starting trees via stepwise addition using the as-is option and branch swapping was performed using nearest neighbor interchange (NNI). MEGA3 was used to average the pairwise patristic distances within and among populations. K2P genetic distances within lineages were compared to distances among lineages using the “Nearest Neighbor Summary” and “Distance Summary” features at the Barcode of Life Data Systems (BOLD) v2.5 website (Ratnasingham and Hebert 2007). These distances were used to calculate Taxonomic Resolution Ratios (TTRs) for *L. ashleyae* and *L. kensleyi*. The TTR is defined as the quotient between mean within lineage distance and mean among lineage distance (Costa et al. 2007).

A likelihood ratio test (LRT) was performed to test for the existence of a molecular clock. Lessios (2008) compiled K2P corrected genetic distances for eight crustacean congener pairs that likely became isolated at the time of final closure of the Isthmus of Panama. Distances ranged from 4.1 to 8.7%, with a mean of 6.75%. Final closure of the Isthmus may have occurred ~2.8 My ago (Schmittner et al. 2004). Combining this date

**Table 3** Mean genetic distances for COI between phylogenetic lineages within the morphospecies *L. ashleyae* and *L. kensleyi* and between morphologically-described amphipod congeners

| Family | | *p* | K2P | GTR |
|---|---|---|---|---|
| | Morphospecies | | | |
| Leucothoidae | Lineages within *L. ashleyae* | 12.4–26.0 | 13.9–32.3 | 15.5–64.9 |
| | Lineages within *L. kensleyi* | 17.0–25.0 | 19.5–30.7 | 26.4–54.2 |
| | Congeners | | | |
| Micruropodinae | *Micruropus crassipes* (AY926680) versus *Micruropus glaber* (AY926682) | 9.9 | 10.9 | 17.1 |
| Iphimediidae | *Echiniphimedia hodgsoni* (AF451350) versus *Echiniphimedia waegelei* (AF451351) | 10.4 | 11.4 | 19.3 |
| Epimeriidae | *Epimeria rubrieques* (AF451345) versus *Epimeria georgiana* (AF451341) | 10.9 | 12.0 | 19.4 |
| Cyamidae | *Cyamus boopis* (DQ095150) versus *Cyamus erraticus* (DQ095128) | 12.4 | 13.8 | 24.2 |
| Eulimnogammarinae | *Eulimnogammarus maacki* (AY926663) versus *Eulimnogammarus viridulus* (AY926665) | 14.4 | 16.2 | 32.2 |
| Gammaridae | *Gammarus oceanicus* (AY926674) versus *Gammarus duebeni* (AF448520) | 17.8 | 20.6 | 51.9 |
| Acanthogammarinae | *Acanthogammarus brevispinus* (AY926651) versus *Acanthogammarus victorii* (AY926652) | 20.8 | 24.8 | 71.6 |
| Eusiridae | *Rhachotropis aculeate* (AY271853) versus *Rhachotropis inflata* (AY271854) | 22.5 | 27.0 | 84.6 |
| Pallaseidae | *Pallasea cancellus* (AY926687) versus *Pallasea grubei* (AY926688) | 22.5 | 27.4 | 90.3 |

GenBank accession numbers are in parenthesis. *p* uncorrected genetic distance, *K2P* Kimura-two parameter corrected genetic distance, *GTR* GTR + G + I patristic distances

with the mean K2P distance (6.75%) produced a sequence divergence rate of 2.4% per Myr, which we used to estimate divergence times between lineages.

## Results

### Haplotype distribution and molecular evolution

We identified a total of 95 distinct haplotypes (*L. ashleyea* = 31; *L. kensleyi* = 64) (Fig. 1). For *L. ashleyae*, the number of haplotypes at each location ranged from 11 (Florida) to 1 (Pelican Cay), and for *L. kensleyi*, the range was 52 (Florida) to 2 (Curaçao). The total number of segregating sites for the region of COI studied was 193, and the substitution ratio for each codon position was 43:18:132 (68.4% at the third position). Rates of synonymous ($d_S$) and nonsynonymous ($d_N$) substitution were 0.484 and 0.045, respectively. The *Z* test showed $d_S$ to be significantly larger than $d_N$ ($P = 0.0001$), indicating that purifying selection was acting on the region of COI sequenced.

Xia's test for substitution saturation calculates a test statistic called an index of substitution saturation ($I_{ss}$), which is compared to its critical value ($I_{ss.c}$). The data set is randomly sampled and $I_{ss}/I_{ss.c}$ are generated from subtrees containing 4, 8, 16, and 32 taxa.

The topology of the tree affects $I_{ss.c}$, (asymmetrical topologies decrease $I_{ss.c}$); therefore, $I_{ss}/I_{ss.c}$ are generated for symmetrical and asymmetrical trees. The critical value of $I_{ss}$ represents the point at which the sequences fail to recover the true tree, so we can be confident that substitution saturation has not occurred if $I_{ss} < I_{ss.c}$ (Xia et al. 2003). The results of the test indicated very little saturation at the third codon position, as all $I_{ss}$ values (except for the symmetrical subtree where $n = 4$) were significantly smaller than $I_{ss.c}$ ($P < 0.001$).

## Phylogenetic analyses

The $\chi^2$ test of homogeneity of base frequencies across taxa showed no significant difference in base composition among lineages ($\chi^2 = 222.8$; $P > 0.999$). Both ML and Bayesian trees described 12 lineages of either *L. ashleyae* or *L. kensleyi* haplotypes, which were further grouped into five major lineages (A–E) (Fig. 1). This perspective on lineages, however, should include the caveat that one of these 12 (1:Vieques) was only a single haplotype and would require additional sampling to verify it as a distinct lineage. Nonetheless, because of its high level of divergence compared to all other members of this dataset, for the purposes of this discussion, it is tentatively considered as one of 12 distinct lineages. With two minor exceptions, Bayesian and ML tree topologies were congruent. In the Bayesian trees, lineages 1, 2, and 3, and lineages 6 and 9 form polytomies. Both ML bootstrap values and Bayesian posterior probabilities provided strong support for the vast majority of lineages (Fig. 1).

With the exception of lineage 6, the geographic distribution of all haplotypes within lineages was restricted to single island locations or Florida. Although lineage 6 was dominated by *L. ashleyae* haplotypes sampled at Carrie Bow Cay, haplotype CB08 ($n = 15$) was observed three times at Pelican Cay and haplotype RO445 was exclusive to Roatan ($n = 14$).

The two major *L. ashleyae* lineages were partitioned geographically, with haplotypes from the west (Belize, Roatan, and Florida) forming lineage C and individuals from the east (Bimini, Vieques, and Curaçao) forming lineage A (Fig. 1). *L. kensleyi* haplotypes showed a similar pattern, with haplotypes from the west (Florida) forming lineage E, while eastern haplotypes formed lineages D (Bimini and Vieques) and B (Curaçao).

Branching order for the five major lineages did not describe reciprocal monophyly for *L. ashleyae* and *L. kensleyi*. However, this branching order had weak statistical support as nodes x, y, and z had low posterior probabilities (in particular node x) and were collapsed in the ML bootstrap consensus tree, which formed a large polytomy of five lineages (indicated with asterisks below branches in Fig. 1). In addition, the SH test indicated that an ML tree constrained to make *L. kensleyi* and *L. ashleyae* monophyletic was not significantly different from the best ML tree ($P = 0.137$). Given these results, we investigated the possibility that amino acid sequences might better capture basal relationships for these taxa. However, although the resulting phylogeny recovered the same five major lineages (tree not shown), branching order among them was again unresolved due to low bootstrap support (all values were below 54%).

## Genetic distance

All 12 lineages were highly divergent, with mean *p* and K2P sequence distances, and mean GTR + G + I patristic distances for the *L. ashleyae* lineages ranging from 12.4 to 26.0%

(median = 21%), 13.9 to 32.3% (median = 24.7%), and 15.5 to 64.9% (median = 43.3%), respectively, (Table 3; Fig. 2). Distances for *L. kensleyi* lineages ranged from 17.0 to 25.0% (median = 22.3%), 19.5 to 30.7% (median = 26.7%), and 26.4 to 54.2% (median = 47.1%). Distances between amphipod congeners ranged from 9.9 to 22.5% (median = 14.4%), 10.9 to 27.4% (median = 16.2%), and 17.1 to 90.3% (median = 32.3) (Table 3).

With the exception of the Glovers Reef–Carrie Bow Cay comparison for *L. ashleyae* (15.5%), all GTR + G + I patristic distances for lineages of both morphospecies exceeded the species delimitation threshold of 16% for COI recently proposed for crustaceans (Lefébure et al. 2006) (see Fig. 2). The *p* and K2P sequence distance medians for *L. ashleyae* and *L. kensleyi* were significantly larger than the medians for the congeners. Mann–Whitney one tail test: *L. ashleyae–p*: $P = 0.015$ (U = 64.5); K2P: $P = 0.018$ (U = 67), *L. kensleyi–p*: $P = 0.033$ (U = 11); K2P: $P = 0.044$ (U = 12).

All mean pairwise K2P distances within lineages (except lineages 3 and 11) were low and ranged from 0.01 to 0.79% (*L. ashleyae*) and 0.13 to 0.84% (*L. kensleyi*). The mean distance within lineages 3 and 11 were 2.94 and 2.40%, respectively. Lineage 3 (*L. ashleyae*; Curaçao) comprised two divergent haplotypes (K2P = 9.4%). Haplotype CR639 was restricted to the northern sampling site (Sunset Reef) and haplotype CR655 to the southern site (Water Factory) (Fig. 1) (27 km separated these sites). As a perspective, the mean pairwise K2P distance between the northern (Palm Beach) and southern (Key West) sites along the Florida coast, separated by 355 km, was 0.5% for *L. ashleyae* and 0.9% for *L. kensleyi*. *L. kensleyi* haplotypes in lineage 11 (all from the same sampling site in Bimini) formed two distinct clades, with a mean pairwise K2P distance of 4.4%.

At both Vieques and Roatan, two highly divergent lineages of haplotypes were observed for *L. ashleyae*. In Vieques, a single haplotype (VQ532) formed lineage 1 which had a mean pairwise K2P distance of 17.3% from the remaining haplotypes that formed lineage

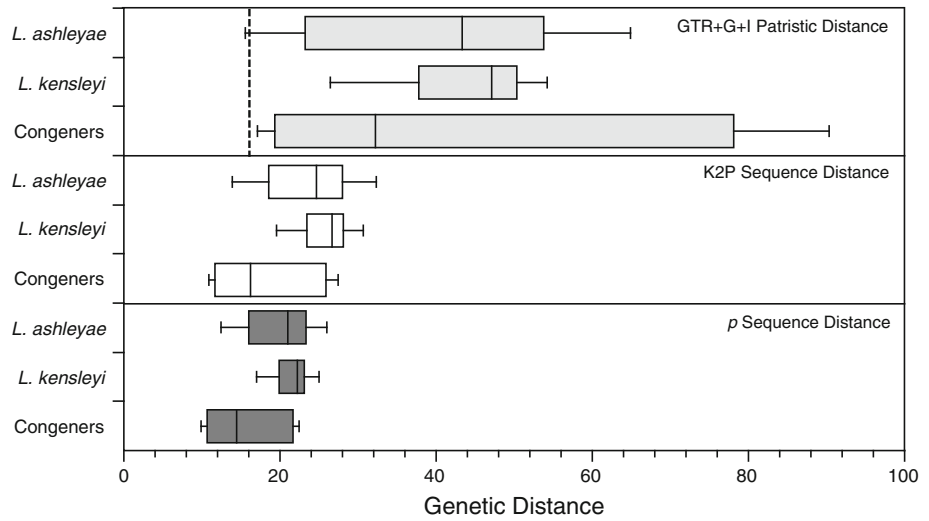


**Fig. 2** Box plot of different distance measures among *L. ashleyae* and *L. kensleyi* lineages and between nine congener pairs. The *upper* and *lower bounds* of each *box* represent the respective quartiles, with the *whiskers* indicating the extremes of the data. The *line* within each *box* denotes the median. The *dashed line* in the *top section* indicates the 16% crustacean species threshold for GTR + G + I patristic distance

4. In Roatan, two haplotypes (lineage 8) were observed at all three sampling sites. However, a third haplotype (RO445), which had a mean pairwise K2P distance from lineage 8 of 18.8%, was exclusive to the patch reef off the southern coast (Smith Bank) and fell within lineage 6 (Carrie Bow Cay).

Including all lineages, the mean within and among lineage K2P distances were 0.54% and 23.9% for *L. ashleyae* and 0.83% and 26.0% for *L. kensleyi*. These distances produced TRRs of 44.5 for *L. ashleyae* and 31.3 for *L. kensleyi*.

The mean of pairwise genetic distances among all individuals between the two morphospecies of *L. ashleyae* and *L. kensleyi* was considerably higher than the mean within each morphospecies: between *L. ashleyae* and *L. kensleyi* = 23.0% (*p*), 27.8% (K2P), 47.2% (patristic); within *L. ashleyae* = 15.9% (*p*), 18.8% (K2P), 31.5% (patristic); within *L. kensleyi* = 9.1% (*p*), 10.8% (K2P), 18.3% (patristic).

The possibility that the large genetic distances reported here were generated through the inadvertent sequencing of nuclear pseudogenes (Buhay 2009) is highly unlikely, since several lines of evidence indicate our data were obtained from a functional gene: (i) absence of aberrant start/stop codons, (ii) typical substitution bias observed at the third codon position, and (iii) purifying selection indicating functional constraint.

## Population genetic diversity

We have documented previously that *L. ashleyae* and *L. kensleyi* show high levels of intraspecific gene flow among Florida reef sampling sites (Richards et al. 2007); consistent with these earlier results, both morphospecies sampled from these Florida sites formed single lineages in the phylogenetic analyses. Consequently, for each morphospecies species, we considered all Florida sampling sites as belonging to a single interbreeding population and grouped them as such in the genetic diversity analysis. The two highly divergent *L. ashleyae* haplotypes from Vieques and Roatan (VQ532 and RO445) were omitted from the analysis (see "Discussion" section).

In general, both *L. ashleyae* and *L. kensleyi* showed much higher levels of genetic diversity for Florida than they did for the island locations (Table 4). However, there were

**Table 4** Measures of genetic diversity

| *L. ashleyae* | | | | | | | *L. kensleyi* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Location | n | H | S | $\pi$ | $\theta_w$ (site) | $\theta_w$ (sequence) | n | H | S | $\pi$ | $\theta_w$ (site) | $\theta_w$ (sequence) |
| Florida | 119 | 10 | 11 | 0.0046 | 0.0050 | 2.06 | 198 | 52 | 53 | 0.0084 | 0.0223 | 9.21 |
| Bimini | 30 | 3 | 8 | 0.0078 | 0.0049 | 2.02 | 14 | 7 | 21 | 0.0233 | 0.0167 | 6.92 |
| Vieques | 30 | 5 | 5 | 0.0039 | 0.0031 | 1.26 | 20 | 3 | 5 | 0.0028 | 0.0034 | 1.41 |
| Curacao | 27 | 2 | 36 | 0.0273 | 0.0226 | 9.34 | 26 | 2 | 2 | 0.0013 | 0.0013 | 0.52 |
| Carrie Bow Cay | 25 | 4 | 3 | 0.0025 | 0.0019 | 0.79 | | | | | | |
| Glovers Reef | 17 | 3 | 2 | 0.0008 | 0.0014 | 0.59 | | | | | | |
| Roatan | 35 | 2 | 1 | 0.0001 | 0.0006 | 0.24 | | | | | | |

*n* Number of samples used in analysis, *H* number of haplotypes, *S* number of segregating sites, $\pi$ nucleotide diversity, $\theta$ population parameter theta

two exceptions to this general trend. First Bimini, where values of nucleotide diversity for *L. ashleyae* and *L. kensleyi* exceeded those for Florida, and second Curaçao, where all values for *L. ashleyae* were very high due to the two divergent haplotypes observed there.

### Molecular clock

The results of the LRT showed that the nucleotide substitution rate was homogeneous across all lineages (test statistic = 64.3; $P = 0.097$). Divergence time for *L. ashleyae* and *L. kensleyi* was 11.6 Myr ago, indicating that these morphospecies split in the mid Miocene.

## Discussion

### Cryptic speciation?

Numerous forms of the phylogenetic species concept have been presented (Avise 2000); however, fundamentally most agree that species are lineages (De Queiroz 1998) and we have identified 11 or 12 genetically highly divergent lineages, which could be characterized as distinct species under a strict interpretation of the phylogenetic species concept of Wheeler and Platnick (2000). However, the delineation of species boundaries using single locus mitochondrial data is controversial (Hickerson et al. 2006), and much of the debate focuses on the utility of COI as a universal "DNA barcode." For some taxonomic groups, the level of intraspecific compared to interspecific variation in this locus may be too high (Moritz and Cicero 2004). Nevertheless, by examining 1,500 COI sequences from 276 different crustacean species, Lefébure et al. (2006) were able to show a clear species delineation threshold for crustaceans of 16% patristic genetic distance. Patristic distances among 11 of the 12 lineages identified here (Carrie Bow Cay–Glovers Reef is borderline) exceed this threshold, providing support for species status for these 11 lineages. However, there is evidence involving amphipods for highly divergent (based on genomic DNA RFLPs) populations, associated with habitat isolation, that are capable of producing viable F1 and F2 hybrids in laboratory crosses (Stanhope et al. 1993). This suggests that amphipods might have an increased rate of molecular evolution, which could render the genetic distance threshold a relatively poor tool for demarcating species in this group of Crustacea. Given this possibility, a more appropriate comparison is to amphipod congeners. Again, species status is supported as the median $p$ and K2P sequence distances exceed those for nine undisputed morphological congeneric species representing nine distinct families. Another comparative measure that should be robust to accelerated rates of mutation is the ratio between intra-lineage and inter-lineage genetic distance (TTR), and the ratios for the study species (*L. ashleyae* = 45; *L. kensleyi* = 31) are comparable to, and often higher than, the ratios reported in other general crustacean COI barcoding surveys (e.g., 18–48—Costa et al. 2007; 9–42—Costa et al. 2009; 25—Radulovici et al. 2009), providing further support for conferring species status to the amphipod lineages.

Despite the very high divergence among lineages, the branching order for the five major lineages is not completely resolved and constraining the tree to support the morphological species concept, in which the two amphipod morphotypes are each monophyletic, cannot be statistically rejected. Thus, we are left with two possible explanations regarding the diversification of these lineages: (i) the two morphotypes are monophyletic, or (ii) the highest likelihood tree (Fig. 1), or something similar to it, is the true tree with several

instances of interspecific convergent evolution in morphology. The highly restricted ability of the amphipods to disperse between islands mirrors that of terrestrial species that inhabit oceanic islands, and convergent evolution on morphology has been observed for species of beetles, spiders, and lizards (see Emerson 2002 for a review). However, for the study species this scenario appears highly unparsimonious since it entails several such convergent events to identical morphologies for both morphospecies for reasons that are entirely unapparent. An additional factor that might be confounding phylogenetic reconstruction of the basal relationships is the possibility that these amphipods experienced an initial period of rapid radiation and diversification (see below for further discussion). Clearly, additional insight into these relationships and also species delineation, where there is always a level of uncertainty, would be gained from future studies that included data from a nuclear locus and additional Leucothoe species.

## Colonization and morphological stasis

Although the actual nucleotide substitution rate for the species studied is unknown, the use of rates published for other crustaceans provide a useful estimate of divergence times. Using these rates, it appears that *L. ashleyae* and *L. kensleyi* lineages diverged in the mid Miocene ($\sim$12 Myr ago) and then colonized separate regions of Florida and the Caribbean. A similar pattern of wide-scale synchronous colonization was also proposed for *Niphargus* groundwater amphipods throughout eastern France (Lefébure et al. 2007). The initial period of radiation for the study species coincides with the scenario proposed by Roth et al. (2000) where the Caribbean was undergoing dramatic transition in the middle to late Miocene (8–12.5 Myr ago). During this period termed the "carbonate crash" (Lyle et al. 1995) large shallow carbonate banks and barrier reefs, which extended from Honduras and Nicuragua to Cuba and Haiti, were foundered opening a connection between the northern and southern Caribbean basins. This in turn initiated the flow of the Caribbean, Loop, and Florida Currents. Gene flow for *L. ashleyae* and *L. kensleyi* is not restricted along shallow coastlines (Richards et al. 2007). Therefore, it's possible that prior to the initiation of these currents, the shallow sea level over the existing carbonate banks facilitated radiation throughout the Caribbean. However, once established, the strong flow of these currents (in particular the Florida Current) and erosion of the carbonate banks creating deeper water could have been responsible for the isolation and independent evolution of clades A and C (*L. ashleyae*) and clades D and E (*L. kensleyi*).

Overall, the major phylogeographic break for *L. ashleyae* and *L. kensleyi* across our sampling sites appears to be at the Florida Straits, adding to the phylogeographic breaks previously identified for a diversity of other reef species in Florida and the greater Caribbean (e.g., the Mona Passage (Taylor and Hellberg 2003, 2006; Baums et al. 2005), the Gulf of Mexico versus wider Caribbean (Rocha et al. 2005), and Florida/Bahamas versus the Caribbean (Lee and Ó Foighil 2005)). Considering that the process of genetic subdivision within species is likely a species-specific interplay between numerous biological and physical factors, it does not seem surprising that multiple phylogenetic breaks exist. However, with study of additional species (in particular the less charismatic and more cryptic taxa) it will be interesting to see if the general phylogeographic pattern revealed is one of multiple species specific breaks or fewer, more general multi-taxa breaks.

If we accept the monophyly of the two morphospecies, the molecular clock calibration indicates that the *L. ashleyae* and *L. kensleyi* morphologies may have been in stasis for $\sim$12 Myr. Witt and Hebert (2000) showed a similar period of morphological stasis for

freshwater *Hyalella* amphipods. A possible explanation for prolonged morphological stasis is that some form of stabilizing selection is acting to restrain morphological character change (Palumbi and Benzie 1991; Wellborn and Broughton 2008). For example, Witt et al. (2003) proposed that fish predation was the ecological factor constraining morphological divergence in the amphipod species complex *Hyalella azteca*. Whereas Macdonald et al. (2005) suggested that the fossorial (burrowing) lifestyle of micruropodid amphipods was responsible for the morphological stasis observed. *Leucothoe ashleyae* and *L. kensleyi* possess morphological adaptations for a filter feeding lifestyle within the canals and spongocoel of sponges (Thomas and Klebba 2007), and the basic sponge body plan has remained virtually unchanged for 580 Myr (Nichols and Wörheide 2005). Therefore, the constant environment inside a sponge host could be a major factor contributing to the stasis of *L. ashleyae* and *L. kensleyi* morphologies.

## Dispersal patterns

In contrast to the unrestricted gene flow observed along the shallow Florida coastline (Richards et al. 2007), the high genetic distance among Caribbean islands indicates that open expanses of deep water have been strong barriers to dispersal for *L. ashleyae* and *L. kensleyi* over evolutionary time scales. Moreover, this barrier to gene flow appears to have been effective over even very short geographic distances. For example, Carrie Bow Cay and Glovers Reef, separated by only 32 km, appear to have been isolated for $\sim$6 Myr. Conversely, individuals at Pelican Cay (18 km south of Carrie Bow Cay) shared identical haplotypes indicating unrestricted gene flow along the shallow Belizean barrier reef system. The lack of a pelagic larval phase seems a likely explanation for this inability to disperse across open expanses of deep water. Similarly, a study of four crustacean and two gastropod species inhabiting Southwest Pacific seamounts detected limited dispersal for the only species that lacked a pelagic dispersal phase (Samadi et al. 2006).

The geographic distribution of the *L. ashleyae* and *L. kensleyi* lineages (mainly restricted to island locations) is analogous to the distribution of groundwater amphipods, which are usually restricted to fresh water springs with a limited ability to disperse among them. These fresh water springs are often described as "aquatic islands," and numerous studies of the evolutionary genetics of groundwater amphipods have revealed a pattern of large genetic divergence among springs (Lefébure et al. 2007; Witt et al. 2006; Finston et al. 2007; Bradford et al. 2010; Murphy et al. 2009; Flot et al. 2010), which mirrors closely the large divergence detected between islands for the *L. ashleyae* and *L. kensleyi* lineages. Similarly, Markow and Pfeiler (2010) showed high genetic divergence among discontinuous coastal habitats for an intertidal isopod with highly restricted dispersal ability.

Although open expanses of deep water appear to be strong barriers to dispersal, they are not impassible, as we detected a few instances of dispersal among island locations for *L. ashleyae*. Lineage 6 (Carrie Bow Cay) provides an example, as 14 individuals all sharing the same haplotype (RO445) from this lineage were observed on the patch reef off Roatan. It seems likely that these haplotypes evolved on the Belizean barrier reef and were subsequently transported to Roatan. If the *L. ashleyae* lineages are separate species, this dispersal represents an example of secondary contact. Although this process is likely to be rare, similar patterns of dispersal have been reported for other species also possessing limited dispersal capabilities and occupying fragmented habitat. For example, Turbinid gastropods on Pacific islands (Meyer et al. 2005), *Niphargus* amphipods within

groundwater (Lefébure et al. 2007), and *Geothelphusa* crabs in freshwater streams and swamps (Okano et al. 2000).

Secondary contact could also explain the occurrence of highly divergent *L. ashleyae* haplotypes in Vieques. Haplotype VQ532 (lineage 1) grouped closely to lineage 2 (Bimini), suggesting that it could have evolved somewhere in the Bahamas and was then transported (possibly over generations) south to Vieques. Although a similar process may have occurred in Curaçao for *L. ashleyae* (lineage 3) it might better be described as admixture. Here, two divergent haplotypes (K2P = 9.4%), separated by shallow water, were observed in close proximity (27 km). Despite being highly divergent, the genetic distance between these haplotypes fell well below the species delineation threshold; consequently, we still consider them as belonging to the same species. The high gene flow consistently observed in other shallow water environments makes it unlikely that these haplotypes diverged in such close proximity in Curaçao. Rather, a more plausible explanation is that, subsequent to their initial split, they have been evolving independently in separate island locations before being brought back into close proximity via recent colonization. A similar pattern was observed on Hawaii for two highly divergent, geographically proximate, lineages of the shrimp *Halocaridina rubra* (Santos 2006).

## Population genetic diversity

Numerous studies of terrestrial species, involving both mitochondrial and nuclear data, have shown lower levels of genetic diversity for island populations when compared to mainland counterparts (Frankham 1996; Paetkau et al. 1998; Eldridge et al. 1999; Stevens and Hogg 2003) highlighting the potentially increased vulnerability of these populations to environmental change. The highly restricted dispersal ability of the study species mirrors that of terrestrial species restricted to island habitat and the levels of genetic diversity detected in this study follow the same pattern. However, results for Curaçao (*L. ashleyae*) and Bimini (*L. ashleyae* and *L. kensleyi*) did not appear to follow this general trend. The high diversity observed in Curaçao can be explained by admixture (as explained above), and removal of one of the divergent haplotypes from the analysis would effectively reduce the diversity to zero. The situation in Bimini is different, as numerous haplotypes were observed for both *L. ashleyae* and *L. kensleyi*. A possible explanation for this finding is that Bimini has not been genetically isolated from surrounding island populations. For example, the shallow waters of the Great Bahama Bank could have united Bimini with numerous other islands and reefs creating a much larger combined effective population size allowing genetic diversity to reach higher levels.

Whether one accepts, or not, the argument regarding high genetic distances between lineages as indicative of distinct species, it is nonetheless the case that within each amphipod morphospecies we have identified multiple ESUs endemic to Florida and Caribbean island locations. The high level of island endemism detected even with our modest geographic sampling adds to the view (Plaisance et al. 2009) that coral reef species and evolutionary diversity, especially that represented by cryptofauna, are likely profoundly underestimated. The high degree of morphological conservatism seen in the amphipods, despite underlying strong genetic divergence, points also to our limited knowledge of the evolutionary history of coral reef ecosystems. Productive allocation of the limited resources available for reef conservation efforts, if aimed at preserving genetic diversity and associated ecosystem resilience to environmental change, will benefit from better information on the magnitude of biodiversity of reef systems.

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