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The Hypervariable Domain of the Mitochondrial Control Region in Atlantic Spiny Lobsters and Its Potential as a Marker for Investigating Phylogeographic Structuring

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Abstract

Atlantic spiny lobsters support major fisheries in northeastern Brazilian waters and in the Caribbean Sea. To avoid reduction in diversity and elimination of distinct stocks, understanding their population dynamics, including structuring of populations and genetic diversity, is critical. We here explore the potential of using the hypervariable domain in the control region of the mitochondrial DNA as a genetic marker to characterize population subdivision in spiny lobsters, using *Panulirus argus* as the species model. The primers designed on the neighboring conserved genes have amplified the entire control region (approx. 780 bases) of *P. argus* and other closely related species. Average nucleotide and haplotype diversity within *P. argus* were found to be high, and population structuring was hypothesized. The data suggest a division of *P. argus* into genetically different phylogeographic groups. The hypervariable domain seems to be useful for determining genetic differentiation of geographically distinct stocks of *P. argus* and other Atlantic spiny lobsters.

Key words: control region — spiny lobster — *Panulirus argus* — mtDNA — genetic structure

Introduction

Atlantic spiny lobsters (Crustacea: Palinuridae) are widely distributed throughout the Atlantic Ocean, the Caribbean Sea and the Gulf of Mexico, and are also found along the African coast (Holthuis, 1991; Cervigon et al., 1993). This species group in general, and *Panulirus argus* in particular, supports one of the most financially valuable fisheries in northeastern Brazilian waters and in the western central Atlantic Ocean. Like many other marine resources, spiny lobsters are experiencing a long-term decline in catch owing to excessive fishing efforts. In recent years a major effort has been directed to the understanding of their population dynamics, including the structure of populations and genetic diversity. Moreover, the influence of marine currents, river outflows such as the Amazon River, and the geomorphology of the coast may favor the geographic isolation of *P. argus* and, consequently, the formation of distinct populations. This information is not only critical in order to avoid loss in diversity, and possibly the elimination of genetically distinct stocks, but also necessary for the elaboration of effective management strategies.

Several different molecular approaches (allozymes, restriction fragment length polymorphism [RFLP], mitochondrial DNA sequencing, microsatellites, etc.) have been applied to assist the study of genetic variability in marine organisms at the population level (Shaklee and Bentzen, 1998). Mitochondrial DNA sequencing, particularly of the most rapidly evolving and highly variable control region, has proved to be a useful tool for population genetic studies of many terrestrial and aquatic organisms (Avise, 1994). The control region, which includes the D-loop in vertebrates, also known as the AT-rich region in invertebrates, does not code for a functional gene and therefore is under fewer functional

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and structural constraints, leading to a high average substitution rate (Saccone et al., 1987). It is usually the fastest evolving region in the mitochondrial DNA of vertebrates and invertebrates, and therefore more sensitive than protein loci as a marker of phylogeographic structuring of many organisms (Caccone et al., 1996; Avise, 2000). Little is known of the structure and evolution of the marine invertebrate mitochondrial control region, but in shrimp it is believed to be divided into 3 polymorphic domains set apart by 2 stretches with no intraspecific variability (Grabowski and Stuck, 1999). Although the mitochondrial control region is a noncoding region, the more conserved parts of it serve as transcriptional promoters and also as a replication origin for one of the mtDNA strands. The peripheral segments, adjacent to flanking coding regions, are highly polymorphic, with a higher base substitution rate than the central domain, and therefore usually termed hypervariable regions and most frequently used as population genetic markers (Grabowski and Stuck, 1999; Grabowski et al., 2004).

Despite the high rates of evolution within the control region of vertebrate mitochondrial genomes and expectations for this region as a sensitive population subdivision detection tool, this may not necessarily hold true for all vertebrates and invertebrates (Zhang and Hewitt, 1997), and should be taken into consideration when a molecular marker is selected for fine scale population genetic studies. It has been reported that the control region may show only modest levels of intraspecific variability in many species, such as fishes (Wilson et al., 1997), butterflies (Taylor et al., 1993), mosquitos (Caccone et al., 1996) locust and grasshopper (Zhang et al., 1995), sea cucumber (Arndt and Smith, 1998), and crustaceans (Straughan and Lehman, 2000), among others. Conversely, the control region has been used with success to study population genetics of other invertebrates (Grabowski and Stuck, 1999; Schultheis et al., 2002; McMillen-Jackson and Bert, 2003; 2004).

The hypervariable domain in the control region of the mtDNA may also help elucidate the taxonomic status of the 2 color morphotypes found in the western Atlantic Ocean, one in the Caribbean and the other in the more southern locations. DNA sequencing of 2 mitochondrial coding genes has uncovered high levels of divergence between *P. argus* ‘stocks’ from the Caribbean Sea and off the Brazilian coast, raising an argument for the separation of the spiny lobster *P. argus* into 2 subspecies or species (Sarver et al., 1998).

The objective of this study was to explore the potential of using the 5′-end hypervariable domain of the control region of the mtDNA, hereafter named HV-CR$_{d1}$, as a genetic marker, for detecting population-level processes in order to characterize population subdivision in spiny lobsters, and to achieve a preliminary assessment of the genetic variability of the Caribbean spiny lobster *P. argus*.

Materials and methods

Sample collection, preservation, and DNA extraction. Specimens of *P. argus* were collected from 8 different localities within the western Atlantic Ocean according to the hypothetical ‘stock’ division detailed by Medley and Venema (2000) and Cochrane and Chakalall (2001) (Figure 1). Samples from other spiny lobster species were obtained from locations given in Table 1. The species *Panulirus japonicus* from the Indo-West Pacific Ocean served as a positive control in polymerase chain reaction (PCR) amplifications, and the remaining species were used to test the versatility of the primers designed for this non-coding region. Tissue samples from walking legs (pereiopods) or antennae were immediately preserved either in 20% dimethylsulfoxide (DMSO) in a saturated sodium chloride solution (Amos and Hoelzel, 1991; Dawson et al., 1998) or in 95% ethanol, and stored at −20°C until further use.

DNA was extracted from the muscle tissue using a phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v) extraction of sodium dodecylsulfate (SDS)–proteinase-K–digested tissue of each individual (Sambrook et al., 1989). High molecular weight DNA was isolated by ethanol precipitation and visualized by gel electrophoresis.

Design of mitochondrial control region primers. Universal control region primers and other primers located on adjacent genes (Kocher et al., 1989; Simon et al., 1994; Palumbi, 1996) were tested on *P. argus* with negative results. Nonamplification or a yield of a large number of nonspecific bands and unsuccessful optimization of reaction conditions were among the main factors observed. Therefore, new control region primers for lobster species were designed. Oligonucleotide primers (Table 2) were designed on more conserved genes that flank the lobster control region using the mitochondrial genome published for *P. japonicus* (GenBank accession number NC004251; Yamauchi et al., 2002).

The forward primer CRL-F was rooted in the small subunit ribosomal RNA (SSU rRNA — 12S) gene, and the reverse primer CRL-R in the isoleucine transfer RNA (tRNA-Ile) gene (Figure 2). Additionally, *P. argus*–specific internal primers were designed to facilitate sequencing. The primer design software PRIMER 3 (Rozen and Skaletsky, 2000) was used for this task. The search for possible secondary struc-

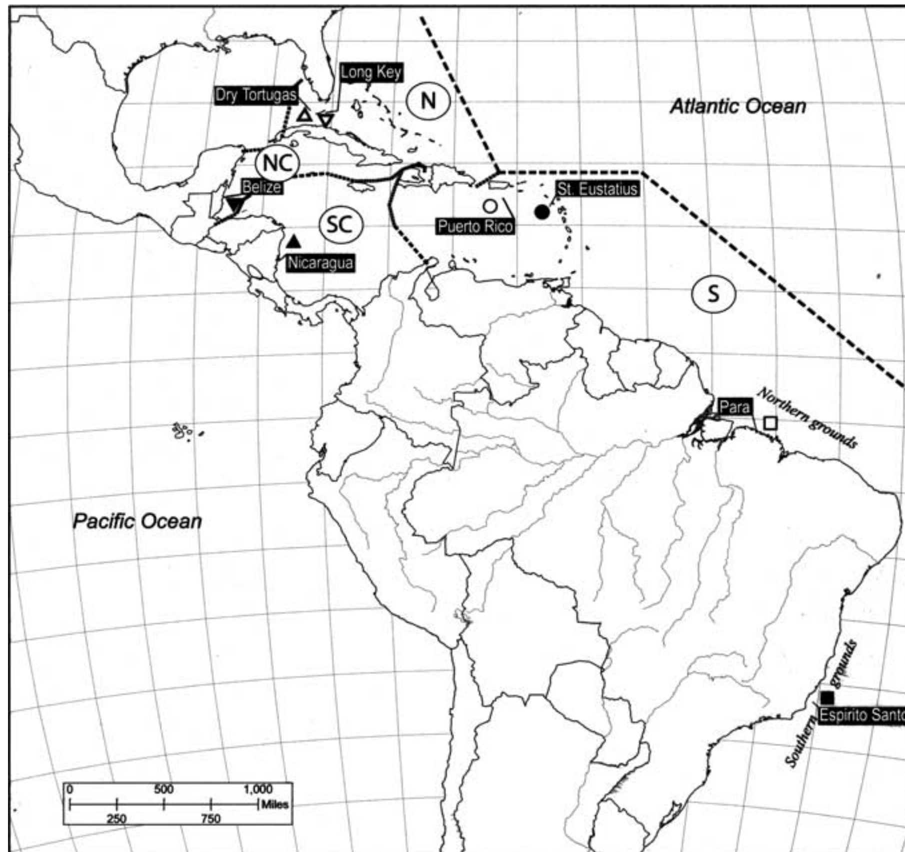

Fig. 1. Sampling locations in the Caribbean Sea and off the Brazilian coast. Symbols represent approximate sites of 'stock' collection and also refer to the haplotypes described on the phylogenetic tree. N indicates Northern; NC, North Central; SC, South Central; and S, Southern. Adapted from Medley and Venema (2000) and Cochrane and Chakalall (2001).

tures such as hairpins and primer-dimers was performed using the computer program GENERUNNER Version 3.05 (Hastings Software).

Control region amplification, purification of PCR products, and sequencing of the HV-CR$_{d1}$. The entire mitochondrial control region of *Panulirus* and *Palinurus* species was amplified using the primers CRL-F and CRL-R via PCR. Each 20 μl PCR contained 20 to 100 ng of the DNA extracted, 3.3 μl of 10× PCR buffer (with $MgCl_2$, 15 mM), 1.6 μl of deoxynucleotide triphosphate mix (dNTPs, 10 mM), 4.0 μl of 5× Q-solution (Qiagen), 1.0 μl each of primer (10 μM), 0.2 μl of *Taq* DNA polymerase (5 U/μl), and sterile HPLC-grade H_2O. Reaction mixes were carried out on a GeneAmp PCR System 2400 thermo-

Table 1. Locality and reference data for specimens from which genomic DNA was extracted

Species (common names)	*General distribution*	*N*	*Stock*[a]	*Collection locality*
Panulirus argus[b] (Caribbean spiny lobster)	Western Atlantic	3	Northern	Long Key, Florida, U.S.A.
		3	Northern	Dry Tortugas, Florida, U.S.A.
		3	South Central	Miskito Cays, Nicaragua
		4	North Central	Belize
		3	Southern	Saint Eustatius
		5	Southern	Puerto Rico
		4	Southern	Brazil, Para State
		3	Southern	Brazil, Espírito Santo State
Panulirus laevicauda (Smoothtail spiny lobster)	NE South America	2	—	Ceará, Brazil
Panulirus guttatus (Spotted spiny lobster)	Caribbean	2	—	Long Key, Florida, USA
Panulirus echinatus (Brown spiny lobster)	Central Atlantic	1	—	Cape Verde
Panulirus japonicus (Japanese spiny lobster)	Japan	1	—	Shikoki Island, Japan
Palinurus delagoae (Natal spiny lobster)	SW Indian Ocean	1	—	Durban, South Africa
Palinurus gilchristi (Southern spiny lobster)	S South Africa	1	—	Port Elizabeth, South Africa
Palinurus elephas (Common spiny lobster)	NE Atlantic	2	—	Italy coast

[a]*P. argus* stocks were hypothesized by Medley and Venema (2000) and Cochrane and Chakalall (2001).
[b]Only the hypervariable domain of the control region (HV-CR$_{d1}$) was sequenced for all *P. argus* individuals.

Table 2. **Oligonucleotide primers designed and used for PCR amplification and sequencing of spiny lobster control region**

Primer	*Oligonucleotide sequences*	*Gene location*	*Primer position*[a] (bp)
CRL-F	5′-GCA AAG AAT ATA GCA AGA ATC AA-3′	SSU rRNA	13493–13517
CRL-R	5′-GCA AAC CTT TTT ATC AGG CAT C-3′	tRNA-Ile	14335–14314
CRPa-F	5′-TCC TTT CAT CAC CAA AAA CTC C-3′	Internal primers for the	
CRPa-R	5′-GCA CGG CTC ACT CTA TCT CC-3′	mitochondrial CR of *P. argus*	

[a]Based on the *P. japonicus* mitochondrial genome.

cycler (PerkinElmer) using the following cycle conditions: 1× 95°C for 10 minutes, 30× (30 seconds at 95°C, 30 seconds at 61°C, 45 seconds at 72°C), and 10 minutes at 72°C. All amplifications were run against a negative control in which all components except DNA template were added. DNA from *P. japonicus* was also used as a positive control in all reactions. Amplicons were checked for correct size and quality by gel electrophoresis.

PCR products were purified with Qiagen Qiaquick PCR Purification columns in order to remove excess primers and nucleotides, and concentrate the amplified PCR fragments. The purified double-stranded amplification products were used as template DNA in sequencing reactions. Cycle-sequencing was performed using the ABI Prism BigDye Ready Mix (Applied Biosystems) and primers CRL-F, CRPa-F, and CRPa-R on a GeneAmp PCR System 2400 thermocycler. The cycle-sequencing products were purified through Qiagen DyeEx Spin kits to remove unreacted fluorescent BigDye Terminators. The sequencing products were analyzed on the ABI Prism Model 377 Automated DNA Sequencer (Applied Biosystems). All PCR products were sequenced in both forward and reverse directions.

Sequence alignment and phylogenetic analyses. Forward and reverse HV-CR$_{d1}$ sequences for each individual were inspected by eye with the aid of sequence editor CHROMAS Version 2.23 (Technelysium Pty Ltd.) and corrected when necessary. Homologous nucleotide sequences from all samples were aligned using the program CLUSTAL X (Thompson et al., 1997) on the sequence alignment editor BIOEDIT (Hall, 1999). Alignments were double-checked by eye and refined manually if needed. All nucleotide HV-CR$_{d1}$ sequences are reported for the first time and were submitted to the GenBank database (National Center for Biotechnology Information).

Estimates of nucleotide sequence divergence between HV-CR$_{d1}$ haplotypes and sampling groups were calculated with the Kimura 2-parameter method (Kimura, 1980). The resulting distances were used to test evolutionary connections among population groups through a neighbor-joining (NJ) tree (Saitou and Nei, 1987) using the program MEGA Version 2.1 (Kumar et al., 2001). Sites with alignment gaps and missing data were omitted. To evaluate significance levels and the consistency of nodes (tree topology) derived from the phylogenetic analysis, 1000 bootstrap replications of the original data set were performed (Felsenstein, 1985). Tree visualization and drawing were carried out using TREEVIEW X Version 0.4.1 (Page, 1996). Statistics of sequence variation, haplotype diversity (H_d), and nucleotide diversity (π) were calculated using the program DNASP Version 4.0.4 (Rozas and Rozas, 1999). The presence of saturation in base substitution for the HV-CR$_{d1}$ region was tested by comparing half of the theoretical saturation index expected when assuming full saturation ($I_{SS.C}$, critical value) with the observed saturation index (I_{SS}) (Xia et al., 2003). Indices of substitution saturation and entropy (H_x), a measure of the amount of variability through haplotype sequences (Schneider and Stephens, 1990), were calculated with the program DAMBE Version 4.2.13 (Xia and Xie, 2001).

Results

HV-CR$_{d1}$ sequence comparisons and statistics. The primers CRL-F and CRL-R amplified the entire control region of several *Panulirus* and *Palinurus* spiny lobsters (Figure 3). The sequence length of

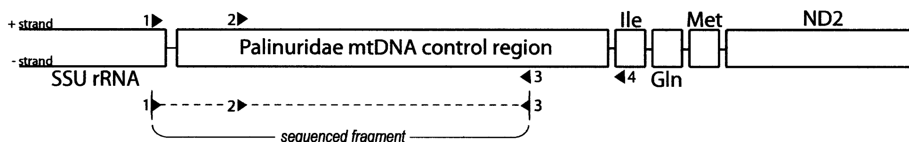

Fig. 2. Position of primers for amplification of the Palinuridae mtDNA control region. Diagram also shows the position of flanking genes at their respective DNA strands. (1►) CRL-F; (2►), CRPa-F; (◄3), CRPa-R; (◄4), CRL-R. SSU rRNA indicates small subunit ribosomal RNA; Ile, transfer RNA–isoleucine; Gln, transfer RNA–glycine; Met, transfer RNA–methionine; and ND2, NADH dehydrogenase subunit 2.

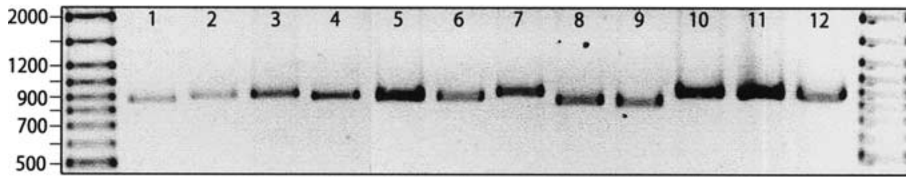

Fig. 3. Inverted tone image of an ethidium-bromide-stained agarose gel, showing PCR products from the amplification of lobster species using the CRL-F:CRL-R primer pair combination. Lane 1, *Palinurus delagoae*; 2-3, *P. elephas*; 4, *P. gilchristi*; 5-6, *Panulirus laevicauda*; 7, *P. japonicus*; 8-9, *P.* guttatus; 10-11, *P. argus*; and 12, *P. echinatus*. Molecular marker: 100-bp DNA ladder (Fermentas).

these Atlantic spiny lobster control regions was estimated by comparison with the ladder and also with the *P. japonicus* PCR band. The Japanese spiny lobster has a control region of 786 bp in length. No size polymorphism or heteroplasmy was observed within or among individuals from the 8 sampled *P. argus* populations. However, distinct amplified control region lengths were detected from different *Panulirus* and *Palinurus* species.

We were able to obtain sequence data from all individuals for a control region fragment of 414 to 417 bp adjacent to the 12S rRNA gene (HV-CR_{d1}). These sequences have been deposited in GenBank under accession numbers AY608716 to AY608739. The nucleotide composition of the hypervariable domain was AT-rich (A+T, 73.2%; C+G, 26.8%); as is usual for this noncoding region in many invertebrates such as insects (range, 68%–75%; Lunt et al., 1996) and crustaceans (range, of 68%–79%; Valverde et al., 1994; Grabowski and Stuck, 1999; McMillen-Jackson and Bert, 2004). A significant difference between the AT-content of HV-CR_{d1} sequences from Caribbean lobsters (73.4%) and Brazilian lobsters (72.4%) was detected at $P < 0.01$ level. Moreover, the average nucleotide composition in the peripheral domain of the control region of *P. argus* is in accordance with the following relationship: A (41.9%) > T (31.3%) > C (16.8%) > G (10.0%).

HV-CR_{d1}* *variability and phylogenetic inferences from hypervariable domain. Table 3 presents the nucleotide sequence alignment of the observed variable sites. Twenty-four unique haplotypes out of the 28 *P. argus* individuals sampled from distinct locations were identified. Different sampling sites did not share a common haplotype. The HV-CR_{d1} sequences contained 160 (38.2%) variable sites, 252 conserved sites, and 7 indels among a total of 419 alignment sites examined. The pattern of nucleotide substitution was biased in favor of transitions over transversions. Variable sites included 32 transitional pairs (s_i, 14 A↔G and 18 T↔C changes) and 25 transversional pairs (s_v, 14 T↔A, 2 T↔G, 6 C↔A, 3 C↔G changes). The estimated s_i/s_v ratio was 1.3. This bias favoring transitions is similar to that reported by other studies of substitutions in mtDNA (Moritz et al., 1987).

The substitution rates do not appear to be higher at the extremities of the hypervariable domain of the control region (Figure 4). A few short stretches (max. length, 16 sites) were found to be nonvariable in the sequence alignment. Site positions 212 to 222, 271 to 286, and 372 to 384 were completely conserved.

Substitution saturation is depicted in Figure 5. Both transitions and transversions are correlated with genetic distances, and at least 96% of the variation in transitions and 99% of the variation in transversions can be explained by a linear regression. Results from the substitution saturation analysis showed an I_{SS} (0.275) much smaller than the critical I_{SS} value ($I_{SS.C} = 0.704$), indicating that sequences are highly useful in phylogenetic reconstruction (Xia et al., 2003).

The magnitudes of haplotype diversity (H_d) and nucleotide diversity (π) were compared between major sampling groups, the Caribbean Sea and the Brazilian coast (Table 4), although small samples sizes may affect the performance of the estimates (Weins and Servedio, 1998). Both estimates were relatively higher for the Caribbean than the Brazilian sampling sites. Average haplotype and nucleotide diversity were respectively 0.928 and 2.6% for the Caribbean Sea, and 0.750 and 0.6% for the Brazilian coast. Overall haplotype diversity was 0.883, while nucleotide diversity was 2.1%.

The genetic distances (Kimura 2-parameter estimates) between sampling groups are given in Table 5. The highest values were found between sites from the Caribbean and Brazil, but in particular between Puerto Rico and Para (NE Caribbean Sea and N Brazil, respectively). The smallest magnitude of this index was found between sampling groups from Saint Eustatius and Puerto Rico, within the Caribbean Sea.

Variable sites were selected for phylogenetic analysis, not including insertions and deletions. Results of inferences of the Kimura 2-parameter (Kimura, 1980) genetic distances, based on nucleo-

Table 3. Variable sites (160 bp) in the hypervariable domain of the mtDNA control region for all *P. argus* unique haplotypes[a]

Haplotypes \ Nucleotide sites	 1111 2467890124	 1111222222 5689012456	 2233345556 7907811230	 6666677777 3456702578	11 8888899900 1278978908	1111111111 1111122222 3456902359	1111111111 3333444455 0125013902	1111111111 5556666677 4560146901
DryTortugas1	CTTTACTGTT	CGAAAATAGT	ATAGCAACCA	TTATACAACA	TACGTTAAAC	AGAATCTACC	TTAGTAATAG	GCCCCCTTTC
DryTortugas2	....	.......G..	....	....	....C.....	....	....	.....T....
LongKey1	.......A..	........A.	....	..G..G....	..G.CA....	.....TC...	.C...CG.T.	..T..T.CC.
LongKey2	.......A..	........A.	....	..G...T...	..G..A....	....	C....C....	.....T....
LongKey3	....	........A.	....	..G.......	..G..A....	....	C....C..G.	.....T....
Nicaragua1	.......A..	........A.	....	..G...T...	..G..A....	....	.....C....	.....T....
Nicaragua2	....	........A.	....	..G...T...	..G..A....	.....T....	C....C....	.....T....
Nicaragua3	....	........A.	....	..G.......	..G..A....	....	C....C..G.	.....T....
Belize1	.......A..	........A.	....	..G...T...	..G..A....	.....A....	C....C....	...T.T....
Belize2	....	........A.	....	..G...T...	..G.......	....	C....C....	.....T....
Belize3	....	........A.	....	..G...T...	..G.......	....	.....C..G.	.....T....
Belize4	..C....A..	........A.	....	..G...T...	..A..A....	....	C....C....	.....T....
PuertoRico1	.....T.A..	.......GA.	.......G..	..G..G....	..G.C.....	.....T....	.C.A.CG.T.	..T..T.CC.
PuertoRico2	.....T.A..	........A.	.......G..	..G..G....	..G.C.G...	.....T....	.C...CG.T.	..T..T.CC.
PuertoRico3	.....T.A..	........A.	.......G..	..G..G....	..G.C.G...	.....T....	.C...CG.T.	..T..T.CC.
PuertoRico4	.....T.A..	........A.	.......G..	..G..G....	..G.C.G...	.....T....	.C...CG.T.	..T..T.CC.
StEustatius1	.....T.A..	........A.	.......G..	C.G..A....	..G.C.G...	.....T....	.C...CG.TA	..T..T.CC.
StEustatius2	.....T.A..	........A.	.......G..	..G..G....	..G.C.G...	.....T....	.C...CG.T.	..T..T.CC.
StEustatius3	.....T.A..	.A......A.	.......G..	C.G..G....	..G.C.G...	.....T....	.C...CG.T.	..T..T.CC.
Para1	TA.A..CCCA	TAGTTTA.AA	TATAAT..TT	.C.ACATGAG	.TACAC.GGT	TAGTCTCCAG	C.T.C.TCT.	TTGTTTA..G
Para2	TA.A..CCCA	TAGTTTA.AA	TATAATG.TT	.C.ACATGAG	.TACAC.GGT	TAGTCTCCAG	C.T.C.TCT.	TTGTTTA..G
Para3	TA.A..CCCA	TAGTTTA.AA	TATAATG.TT	.C.ACATGAG	.TACAC.GGT	TAGTCTCCAG	C.T.C.TCT.	TTGTTTA..G
EspiritoSanto1	TA.AG..TCA	TAGTTTA.AA	TATAAT..TT	.C.ACATG.G	ATACAC.GGT	TAGTCTCTAG	C.T.CGTCG.	A.GTTTA..G
EspiritoSanto2	TA.AG..TCA	TAGTTTA.AA	TATAAT..TT	.C.ACATG.G	ATACAC.GGT	TAGTCTCTAG	C.T.CGTCG.	A.GTTTA..G

Haplotypes \ Nucleotide sites	1111111111 7788888899 4501378901	1111222222 9999001122 2456680137	2222222222 2344455666 8814856028	2222222233 7888999900 0789478905	3333333333 0111222223 8458234572	3333333333 3333444455 5678457923	3333333333 5555566678 4568913415	3333444444 8899001111 6937480468
DryTortugas1	CTAGTCCTCC	AGTATTTCTT	TTGCTTAATA	AAATGCCACG	AGAGGTAAGA	CTAATATCAT	CTACGGAAGT	CCAATGCACG
DryTortugas2	.........T	....	....	.....T....	....	C	....	........T.
LongKey1	.CGA..T..T	..AG..A...	C.........	....AT..TA	......T.A.	.A.GA...GC	TC......A.	...G....T.
LongKey2	.........T	....	....	.........A	......T...	C	....	...G....T.
LongKey3	.........T	.......AA.	C.........	.........A	......T...	......G.G.	.CT......C	...GA...TA
Nicaragua1	.........T	....	....	....A....A	......T...	C	.......G..	........T.
Nicaragua2	........TT	....	..A.......	.........A	......T...	....	....	........T.
Nicaragua3	.........T	....	..A.......	.........A	......T...	..G.......	.CT.......	TT......T.
Belize1	........AT	....	C.........	....A..G.A	......T...	C	....	...G....T.
Belize2	.........T	....	C.........	.........A	......T...	....	....A.....	........T.
Belize3	.........T	....	....	.......G.A	......C...	....	....	........T.
Belize4	.........T	C	....	....A....A	......T...	C	....	........T.
PuertoRico1	.CGA..T..T	..AG..A...	C.........	...AAT..TA	......T.A.	.A.GA...GC	TC.......C	..GG....T.
PuertoRico2	TC.A..T..T	..A...A...	C.........	...AAT..TA	......T.A.	.A.GA...GC	TCG.......	...G......
PuertoRico3	TCGA..TC.T	..A...A...	....	...AAT..TA	......T.A.	.A.GA...GC	TCG.......	...G......
PuertoRico4	.C.A..T..T	..A...A...	C.........	..GAAT..TA	......T.A.	.A.GA...GC	TCG.......	...G....T.
StEustatius1	.C.A..T..T	..A...A...	C.A.......	...AAT..TA	......T.A.	.A.GAG..GC	TCG.......	...G.A..T.
StEustatius2	TC.A..T..T	..A...A...	....	...AAT..TA	......T.A.	.A.GA...GC	TCG.......	...G......
StEustatius3	.C.A..T..T	..A...A...	C.........	...AAT..TA	......T.A.	.A.GA...GC	TCG.......	...G.A..T.
Para1	..TAA...TT	CTC..CG...	.AATCCGTCT	CCCCTTA.AA	TAGAACTCAG	T..GAC.A.G	.AGTACT...	......TGG.
Para2	..TAAT..TT	CTC.CCG...	.AATCCGTCT	CCCCTTA.A.	TAGAACTCAG	T..GAC.A.G	.A.TACT...	...G..TGG.
Para3	..TAA...TT	CTC.CCG...	.AATCCGTCT	CCCCTTA.AA	TAGAACTCAG	T..GAC.A.G	.A.TACT...	......TGG.
EspiritoSanto1	..TAA...TT	CTC.CCA...	.AATCCGTC.	CCC.TTG.A.	TAGAACTCAG	...GAC.AG.	.GGTACT...	...G..TGG.
EspiritoSanto2	.CTAA...TT	CTC.CCA...	.AATCCGTC.	CCC.TTA.A.	TAGAACTCAG	...GAC.AG.	.GGTACT...	...G..TGG.

[a]The dots represent the same nucleotide as occurs in the first sequence for that site. Site numbers are written vertically and indicate only polymorphic positions in the haplotype sequences.

tide divergence of unique haplotypes throughout sampling groups (data not shown), revealed divergence values ranging from 0.002 (distance between haplotypes *PuertoRico2* and *StEustatius2*) to 0.395 (*Para3*, Brazilian haplotype; and *PuertoRico1*, Caribbean haplotype), with an average value of 0.166.

A significant level of phylogenetic signal was detected in the control region data set, showing clear evolutionary connections between sampling groups and locations (Figure 6). The NJ method of phylogenetic reconstruction recovered 2 major evolutionary lineages among haplotypes of *Panulirus argus* with high bootstrap support. In general, different sampling locations were distinguished by groups of closely related haplotypes. Differences were, therefore, population/"stock"-specific in both major clades. The level of sequence divergence was consistently higher from individuals of the Brazilian

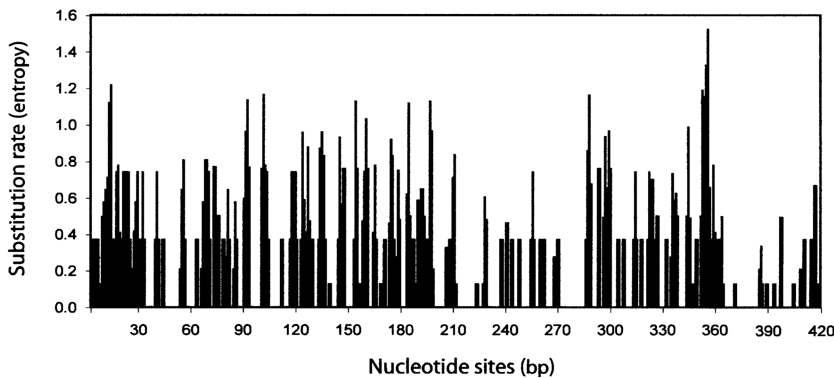

Fig. 4. Distribution of sequence variability (entropy plots) across the hypervariable domain of the mtDNA control region of the spiny lobster *P. argus*. Bars represent the number of variable sites measured in a sliding window of 2 bp.

lineage to the main Caribbean cluster. Internal nodes within both the Caribbean and Brazilian lineages were also highly supported by the 1000-replication bootstrap.

Discussion

Control region sequences have been successfully used previously as informative markers to detect population structure in marine invertebrates, but mostly shrimp species (Chu et al., 2003; McMillen-Jackson and Bert, 2003, 2004). Despite the potential usefulness of the mtDNA control region for studying population subdivision of marine decapods, this noncoding region has never been examined in lobster population genetic studies, the lack of primer sets being one major reason.

A newly designed primer set (CRL-F and CRL-R) amplifying a hypervariable domain at the 5′-end of the mitochondrial control region (HV-CR_{d1}) in spiny lobsters (*Panulirus* and *Palinurus*) is reported. The feasibility of HV-CR_{d1} as a molecular marker for examining the population structure in lobsters (i.e., *Panulirus argus*) is explored for the first time in this study.

The versatility of this primer pair was further characterized by the amplification of homologous fragments from other *Panulirus* species (*P. laevicauda*, *P. guttatus*, *P. echinatus*, and *P. japonicus*) and *Palinurus* species (*P. delagoae*, *P. gilchristi*, *P. elephas*) under the same PCR conditions. Successful amplifications indicate its applicability in a wide variety of spiny lobsters, thus reducing time, effort, and resources on population genetic and phylogeographic studies using the mitochondrial control region as a polymorphic marker. Some slight dissimilarities within control region lengths were detected from different *Panulirus* and *Palinurus* species. Sequences may vary in length because of the presence of imperfect tandem repeats and mononucleotide stretches in these spiny lobsters, which are typically found in the mitochondrial control region of several organisms (Lee et al., 1995; Zhang and Hewitt, 1997; Brzuzan, 2000; Schultheis et al., 2002).

The HV-CR_{d1} in *P. argus* was found to be highly polymorphic, a common feature of this noncoding region in most decapod species studied so far (Grabowski and Stuck, 1999; Chu et al., 2003; McMillen-Jackson and Bert, 2003; 2004; Grabowski et al., 2004). In the HV-CR_{d1} the variable nucleotide sites were distributed randomly along the 5′-peripheral domain within haplotypes. The geographic patterns of nucleotide substitutions seem to be indicative of broad phylogeographic groups (Table 3).

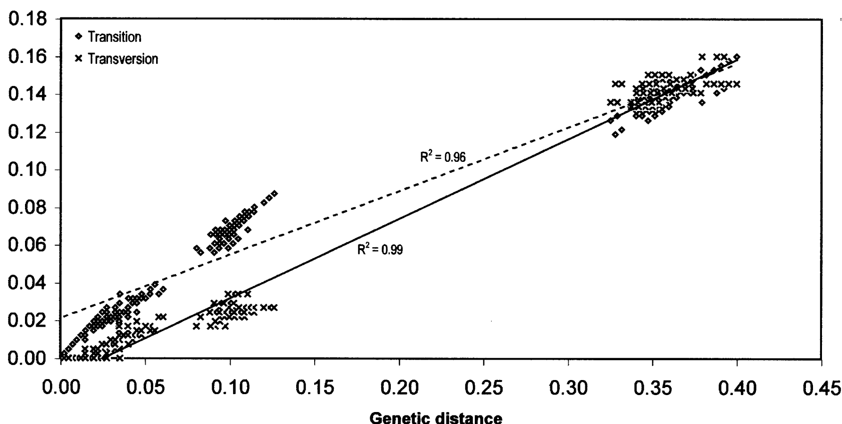

Fig. 5. Saturation plot for the hypervariable domain of the mitochondrial control region in the spiny lobster *P. argus*. Transitions and transversions of the HV-CR_{d1} sequences were plotted against the Kimura 2-parameter–corrected genetic distances.

Table 4. Main characteristics of nucleotide sequences and measures of genetic diversity based on the hypervariable domain of mitochondrial control region of 8 sampling sites of *Panulirus argus*

Populations and sampling sites[a]	*A+T (%)*[b]	*Number of haplotypes (seqs)*	*Haplotype diversity (H_d)*	*Nucleotide diversity (π)*
Caribbean Sea (CS)				
Dry Tortugas	73.3	2 (3)	0.667	0.011
Long Key	73.0	3 (3)	1.000	0.066
Nicaragua	73.9	3 (3)	1.000	0.024
Belize	73.6	4 (4)	1.000	0.023
Puerto Rico	73.1	4 (5)	0.900	0.014
St. Eustatius	73.7	3 (3)	1.000	0.016
$\bar{X}_{CS}$	73.4	-	0.928	0.026
Brazilian coast (BC)				
Para	73.0	3 (4)	0.833	0.009
Espírito Santo	71.8	2 (3)	0.667	0.003
$\bar{X}_{BC}$	72.4	-	0.750	0.006

[a] $\bar{X}$ indicates average among sampling sites within major groups.
[b] A+T indicates average for adenine and thymine content within the population.

A large number of distinct and nonoverlapping haplotypes throughout the studied region were found among sampling sites. However, these apparent patterns are provisional, given the small sample sizes. The high mutation rate inherent in this mtDNA region leads to a fast average substitution rate as found in several marine decapods (Chu et al., 2003) including the studied Palinuridae. Even though such a feature may facilitate the reconstruction of evolutionary relationships among geographically separated groups, it might also represent a problem if the same mtDNA fragment is selected for examination of very distant taxonomic relationships, owing to saturation in base substitution and ambiguities in alignment of homologous sequences. These could misdirect phylogenetic reconstruction (Yang, 1998). Despite the high levels of divergence among *P. argus* haplotypes, the analysis of substitution saturation indices supported the utility of the HV-CR$_{d1}$ region for reconstructing their phylogeographic relationships. The substitution saturation index (I_{SS}) was substantially smaller than its critical value ($I_{SS.C}$), indicating that saturation is not a problem for assessing intraspecific connections. Lack of substitution saturation was also inferred from linearity in plots of transitions or transversions relative to genetic distance.

The number of more polymorphic sites and distinct haplotypes from the HV-CR$_{d1}$ compared with coding DNA regions and restriction fragment length polymorphisms substantiate the use of the control region for evaluating intrapopulational genetic variations (Chu et al., 2003). This study clearly demonstrates that the use of the HV-CR$_{d1}$ of *P. argus* considerably increases the number of informative characters and the resolution power in population differentiation. The levels of divergence seen for the HV-CR$_{d1}$ sequences in *P. argus* are higher than in most studies, if nucleotide divergence between Brazilian sampling sites is compared with the Caribbean's (max. 38.5%). However, if divergence is focused within each Atlantic Ocean region separately, then the divergence magnitude decreases considerably. The highest levels were 11.2% and 5.4% within the Caribbean Sea and off the Brazilian coast, respectively. These values are much closer to those obtained by population genetics of shrimp species (*Farfantepenaeus aztecus*, *F. setiferus*, and *F. duorarum*, max. 6%) sampled from Texas to the western Atlantic coast

Table 5. Average Kimura 2-parameter genetic distances estimated from the hypervariable domain of the mtDNA control region of *P. argus* sampling sites across the Atlantic Ocean

	Dry Tortugas	*Long Key*	*Nicaragua*	*Belize*	*Puerto Rico*	*St.Eustatius*	*Para*	*Espírito Santo*
Dry Tortugas								
Long Key	0.067							
Nicaragua	0.040	0.049						
Belize	0.039	0.049	0.024					
Puerto Rico	0.109	0.075	0.101	0.100				
StEustatius	0.112	0.079	0.101	0.101	0.018			
Para	0.360	0.368	0.350	0.352	0.385	0.382		
Espírito Santo	0.353	0.348	0.342	0.344	0.362	0.358	0.054	

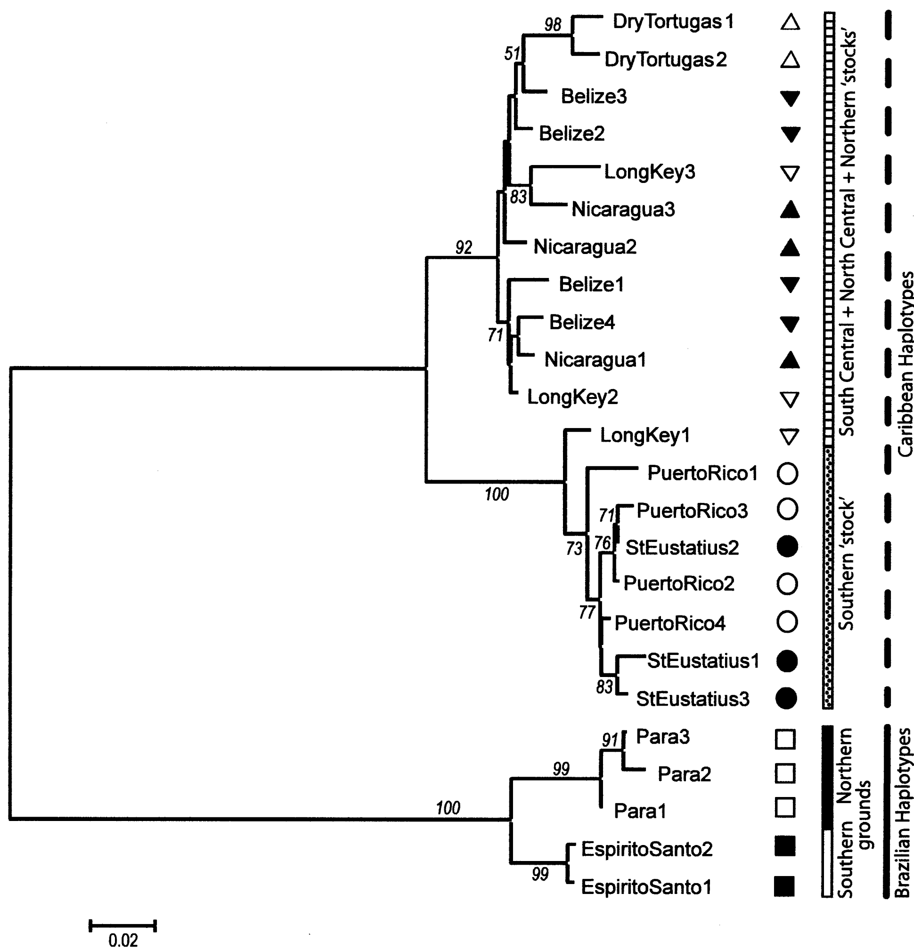

Fig. 6. Neighbor-joining tree describing evolutionary relationships among 24 *P. argus* haplotypes resolved on the basis of the HV-CR$_{d1}$ (419 bp) sequence data. Bootstrap values based on 1000 replicates are indicated only for nodes > 50%.

of the United States (Grabowski and Stuck, 1999; McMillen-Jackson and Bert, 2003; 2004; Grabowski et al., 2004), but lower than the sequence divergence found for *Penaeus merguiensis* between populations in Australia and in China (d = 14.6%; Chu et al., 2003). These authors also report divergence levels of up to 42.1% when different shrimp species are compared. Therefore, if we assume that the distinct Caribbean and Brazilian lineages (Figure 6) are indeed representative of 2 different subspecies or species as claimed by Sarver et al. (1998), then the magnitude of sequence divergence for this spiny lobster seems biologically plausible. Thus, the divergence level between Caribbean and Brazilian lobsters is consistent with subspecies or species variation. It is nonetheless possible that the divergence within groups from the same major sampling areas (Caribbean and Brazil) are slightly underestimated owing to the relatively small number of haplotypes investigated.

The phylogenetic tree produced from the HV-CR$_{d1}$ variation on *P. argus* from the 8 sampling sites rendered high bootstrap support (>92%, 1000 replicates) for all major clades, indicating that the data consistently support evolutionary associations (Hillis and Bull, 1993). A similar tree topology was also recovered for another marine species with a very broad distribution, from NE Brazil to the eastern Caribbean Sea. Gomes et al. (1999) reported an UPGMA phenogram showing similar patterns of geographic distribution and genetic relatedness among 6 sampling groups of flying fish (*Hirundichthys affinis*) in the western central Atlantic.

The presence of the 2 major lineages supported by a 100% bootstrap proportion showing strong molecular genetic structure is probably due to restricted gene flow and the presence of marine barriers to dispersal in the western Atlantic, such as the Amazon River's massive outflow, and current patterns in the region, resulting in the high genetic divergence between the Caribbean and Brazilian morphotypes. The western tropical Atlantic is characterized by numerous gyres, eddies, and seasonally varying currents (Lyons, 1981). The information gained from the control region data, therefore, corroborates the need for more extensive taxonomic status revision of this valuable marine resource. Notwithstanding the reduced sample size,

2 distinct highly supported subgroupings of haplotypes with phylogenetic concordance between sequence type and geographic location were resolved within the Caribbean Sea. No evidence for additional within-group subdivision was observed.

The Belizean, Floridian (Dry Tortugas and Long Key) and Nicaraguan haplotypes were clustered within the same highly supported (92%) clade, with the exception of one haplotype from Long Key. The significance of the presence of the haplotype *Long-Key1* in a different clade is not clearly understood; however, it might well be a relic of an older population with wider distribution (McMillen-Jackson and Bert, 2003). Additionally, Puerto Rico and St. Eustatius, NE Caribbean islands, clustered together with 100% statistical support. The revealed tree topology indicates the possibility of 2 reproductively isolated subpopulations in the Caribbean Sea, with apparently high levels of gene flow within each subgroup. However, it remains to be seen if these population structure patterns are realistic.

The subdivision of the Brazilian form of *P. argus* into 2 distinct lineages must also be viewed with caution. Only small numbers were sequenced from each location off the Brazilian coast, and the sampled haplotypes may not be fully representative of each 'stock' unit. However, the possibility that this genetic distinction may persist with a larger sample size is suggested by the pattern of water currents in the area. The South Equatorial Current bifurcation into the north flowing North Brazil Current and the south flowing Brazil Current may play an important role in maintaining separation of lobster larvae originating from 2 isolated "stock" units (Chekunova, 1972; Fonteles-Filho, 1992; Schott et al., 1998; Epifanio and Garvine, 2001).

In conclusion, our study provides a basis for the use of the hypervariable domain of the mitochondrial control region (HV-CR$_{d1}$) in intraspecific population-level variability studies. The first control region primers for *Panulirus* and *Palinurus* species were also introduced. Our results suggest that the HV-CR$_{d1}$ is an effective marker for revealing the phylogeographic structure of *P. argus* in the Atlantic Ocean waters, and this can be seen as a model for other spiny lobster species in population genetic studies. Very high geographic specificity of *P. argus* haplotypes was noticeable among sampling groups in the Caribbean and Brazilian coast. An indication of population subdivision and genetic differentiation in the spiny lobster *P. argus* was evidenced by the magnitude of its control region sequence variation. The important observation here is that recently evolved population subdivision in species with strong larval dispersal, such as Palinurids, can only be detected with rapidly evolving DNA markers. The data generated by this study also indicate that the assumption of panmixis for the *P. argus* population throughout its range should be reevaluated with further research using the same HV-CR$_{d1}$ marker, but larger sample size and more widespread sampling throughout the distribution of *P. argus*, also in conjunction with polymorphic nuclear markers such as microsatellites.

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