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Distribution, prevalence, and genetic analysis of *Panulirus argus* virus 1 (PaV1) from the Caribbean Sea

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ABSTRACT: The pathogenic virus *Panulirus argus* virus 1 (PaV1) was first discovered in Caribbean spiny lobsters *Panulirus argus* from the Florida Keys (USA) in 1999 and has since been reported in Belize, Mexico, and Cuba; its distribution in the wider Caribbean is unknown. We collected tissue samples from adult spiny lobsters from 30 locations in 14 countries bordering the Caribbean Sea and used molecular diagnostics to assay for the presence of PaV1. PaV1 occurred primarily in the northern areas of the Caribbean, where its prevalence was highest. The virus was not found in lobsters from the southeastern Caribbean, and its prevalence was lowest in the southwestern Caribbean. DNA sequence analysis was performed on a fragment of the viral DNA to examine the genetic diversity of PaV1 on a Caribbean-wide scale. Sequence variation in the viral DNA fragment was high, with 61 unique alleles identified from 9 areas. The sharing of viral alleles in lobsters from distant locations supports the hypothesis of a strong genetic connectivity among lobsters within the Caribbean, and further supports the hypothesis that postlarvae infected with PaV1 may serve to disperse the virus over long distances.

KEY WORDS: Spiny lobster · Disease · Epidemiology · Connectivity

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INTRODUCTION

The Caribbean spiny lobster *Panulirus argus* is abundant throughout the Caribbean Sea and Gulf of Mexico, where it is widely fished, both commercially and recreationally. However, *P. argus* is also found in the northwestern Atlantic (e.g. North Carolina, USA, and Bermuda), and a few vagrants have been identified from as far away as western Europe and Africa (Freitas & Castro 2005). Over the period 1987 to 2001,

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the commercial harvest of *P. argus* in Florida, USA, totaled 42 million kg, valued at US$365.3 million (Florida Wildlife and Conservation Commission 2012). In 2010, more than 35 000 metric tons (t) of *P. argus* were landed worldwide (FAO Fisheries and Aquaculture Information and Statistics Service). Countries with the highest landings include Brazil, the Bahamas, Cuba, Nicaragua, Honduras, the Dominican Republic, and the USA (Vondruska 2010).

In 1999, a pathogenic virus, *Panulirus argus* virus 1 (PaV1), was discovered infecting spiny lobsters from the Florida Keys (Shields & Behringer 2004). Prevalence of PaV1 is highest in early benthic juveniles (EBJs) and lower among larger juveniles and adults (Shields & Behringer 2004, Butler et al. 2008, Lozano-Álvarez et al. 2008). Lobsters with clinical disease have chalky-white hemolymph and often have a discolored carapace with a pink hue. Heavily infected lobsters are typically lethargic, cease grooming and foraging, and have lower hemolymph serum protein values compared to uninfected lobsters (Behringer et al. 2008). Histologically, signs of PaV1 infection include a depletion of the reserve inclusion cells, hemocyte infiltration into the intertubular spaces of the hepatopancreas, focal necrosis of the hepatopancreas, and the presence of infected hemocytes in the heart, gills, and connective tissues (Li et al. 2008).

Since its initial discovery in Florida, infections have been confirmed in Belize (Huchin-Mian et al. 2009), Mexico (Huchin-Mian et al. 2008), Cuba (Cruz Quintana et al. 2011), and the US Virgin Islands (Butler et al. 2008). Prevalence of PaV1 in juvenile lobsters in Florida is patchy, but has historically ranged from 5 to 8%, with some locations exceeding 60% (Behringer et al. 2011). While typically asymptomatic, adult lobsters can be infected by PaV1, as determined through PCR screening of adults in Florida (prevalence: 11%; Behringer et al. 2012), Belize (prevalence in frozen tails: 50%; Huchin-Mian et al. 2009), and Cuba (prevalence: 0.95%; Cruz Quintana et al. 2011).

Inspection of lobster hemolymph viewed through the juncture of the abdomen and ventral carapace is still commonly used to diagnose PaV1 infections, but that method only reveals the most severely diseased individuals whose hemolymph is white. Animals with light and moderate infections cannot be diagnosed visually, and adult lobsters are typically asymptomatic. An assay using PCR is an accurate and sensitive diagnostic method for PaV1 that amplifies an approximately 500 bp viral DNA fragment (GenBank accession no. EF206313) (Montgomery-Fullerton et al. 2007). Molecular detection of viral DNA using PCR has been used to diagnose PaV1 in *Panulirus argus* from Florida (Behringer et al. 2011, 2012, Moss et al. 2012), Mexico (Huchin-Mian et al. 2008), Belize (Huchin-Mian et al. 2009), and Cuba (Cruz Quintana et al. 2011). Sequencing of the 500 bp fragment of viral DNA has revealed high genetic variation in PaV1 from postlarval and juvenile lobsters collected throughout the Florida Keys (Moss et al. 2012).

Due to the relatively recent discovery of PaV1 in adult lobsters in Belize, Cuba, and Florida (Huchin-Mian et al. 2009, Cruz Quintana et al. 2011, Behringer et al. 2012), in addition to our recent discovery of genetic variability in the virus (Moss et al. 2012), we undertook an extensive survey to assess the distribution, prevalence, and genetic variation of PaV1 in adult spiny lobsters from around the Caribbean Sea. Our objectives were to assess the prevalence of the virus in adults from countries around the Caribbean, to examine the genetic diversity of the virus from different regions, and to examine patterns in the diversity of the virus in relation to the spatial connectivity of regions within the Caribbean.

MATERIALS AND METHODS

Sample collection

Hemolymph samples from sublegal- and legal-sized (carapace length ≥75 mm) *Panulirus argus* were obtained during 2008 and 2009 onboard commercial fishing vessels in Florida (see Behringer et al. 2011 for full details). Approximately 200 µl of hemolymph was withdrawn using a sterile syringe inserted at the base of the 5th walking leg and dispensed into 600 µl of sterile sodium-citrate lobster anticoagulant (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA; pH 4.6 to 5.4; Söderhäll & Smith 1983), then frozen at −80°C.

From September 2010 through October 2011, adult *Panulirus argus* tissues were obtained directly from fishermen as fresh or recently frozen tissue samples (5th walking leg) from locations throughout the Caribbean (Fig. 1, Table 1). In most cases, approximately 100 lobsters were sampled from each country or sample location (see Table 1), and carapace length and sex were taken for each lobster. Samples, which consisted of a 1–2 cm piece of a walking leg preserved in clear rum (40% ethanol, 80 proof), were shipped to the Virginia Institute of Marine Science and transferred upon receipt into 95% ethanol for long-term storage in preparation for genetic analysis.

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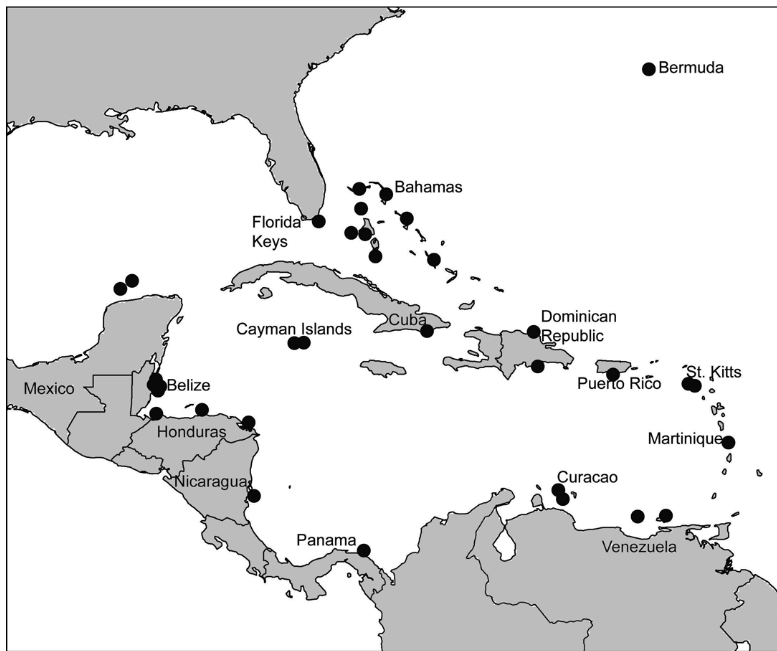

Fig. 1. Sampling locations (●) for *Panulirus argus*

Molecular diagnostics and DNA sequencing

Genomic DNA was extracted using Chelex resin (Bio-Rad). Using sterile methods, aliquots of ~15 mg of tissue or 150 µl of hemolymph in anticoagulant were incubated with 50 µl of 10% Chelex resin (w/v) and 12 µl of Proteinase K (Qiagen) at 60°C for approximately 4 h. Once tissue lysis was complete, samples were vortexed for 15 s, heated at 100°C for 10 min, vortexed briefly again, and then centrifuged at 13600 rpm (20000 × *g*) for 1 min. The DNA (supernatant) was removed and stored at either –20°C or 4°C prior to genetic analysis.

For quality control, genomic DNA from each lobster was assessed by amplifying the small subunit ribosomal RNA (SSU) using 'universal' SSU primers modified from Medlin et al. (1988) (see Moss et al. 2006, 2012). The amplified target DNA fragment was approximately 1800 bp in length.

Diagnosis of PaV1 was done using PCR primers published previously (Montgomery-Fullerton et al. 2007), with modifications to reagent concentrations, thermocycling parameters, and reaction volume as described in Moss et al. (2012). The concentrations of the assay reagents were 1× PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), 0.2 mg ml^{-1} bovine serum albumin (BSA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM 45aF (forward primer), 0.5 µM 543aR (reverse primer), and 1.0 unit *Taq* polymerase (Invitrogen). Aliquots of 2.5 µl of lobster genomic DNA were added to 22.5 µl of PCR reagents for a final PCR volume of 25 µl. An additional control reaction containing 2.5 µl of double-distilled water plus PCR reagents in the above concentrations was included as a separate control (no DNA). The thermocycling parameters were an initial denaturation step at 94°C for 5 min, followed by 40 cycles of 94°C for 45 s, 63°C for 45 s, 72°C for 1 min, all followed by a final elongation step at 72°C for 10 min. Aliquots of 10 µl of the PaV1 PCR product or 6 µl of the SSU product were loaded onto a 2% agarose gel (w/v), electrophoresed at 100 V, stained with ethidium bromide, and examined under UV light. Images were recorded using the Alpha Innotech FluorChem® imaging system. We determined previously that the sensitivity of the modified PCR protocol is 0.02 fg µl^{-1} viral DNA or 0.05 fg of viral DNA per PCR reaction using plasmid DNA of the viral sequence (Moss et al. 2012).

Amplification products from all spiny lobsters found to be PCR-positive for PaV1 were cloned and sequenced. Briefly, between 4 and 8 clones were sequenced per sample. PCR products of the correct size (~500 bp) were cloned into the plasmid pCR®4-TOPO and then transformed into *Escherichia coli* using a TOPO TA Cloning® Kit (Invitrogen), using half the manufacturer's recommendation of vector and chemically competent cells. Selection was based on ampicillin resistance. Transformed bacterial colonies were screened for inserts using a boil-prep method followed by PCR amplification using the M13 vector primers (Moss et al. 2006). Before sequencing, PCR products were treated with shrimp alkaline phosphatase (SAP) and exonuclease I (Exo I) (Amersham Biosciences) to remove excess oligonucleotides and dNTPs. Bidirectional sequencing was performed using the Big Dye Terminator Kit v.3.1 (Applied Biosystems) with M13 sequencing primers in 5 µl reactions, with 1/8 the concentration of Big Dye recommended by the manufacturer's protocols. Sequence products were re-suspended in 20 µl of Hi-Di formamide (Applied Biosystems), and 10 µl of each were electrophoretically separated on an ABI 3130 Prism Genetic Analyzer.

Vector trimming and sequence editing were performed using CodonCode Aligner v.3.7.1.1. Sequences were aligned using the ClustalW algorithm in MacVector v.12.5.1. Settings for pairwise alignment were an open gap penalty of 10.0 and an extend gap

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Table 1. *Panulirus argus* virus 1 (PaV1) infecting *P. argus.* Location and dates where adult lobsters were sampled. Tissue samples are from different individuals; prevalence: percentage of lobsters that were PCR positive relative to the total number sampled. Totals in **bold**

Country	Location	Date sampled	No. of samples	Prevalence (%)	95% CI
Bahamas	Abaco–Sandy Point	Oct 2010	98	0	0.0–3.9
	Andros–Fresh Creek	Oct 2010	77	0	0.0–4.9
	Berry Islands	Nov 2010	106	0.9	0.1–5.2
	Grand Bahama–West End	Nov 2010	99	1.0	0.2–5.6
	Great Bahama Bank–Guanchos Cay	Oct 2010	104	0	0.0–3.6
	Long Island	Oct 2010	93	0	0.0–4.0
	New Providence–Eleuthra Bank	Nov 2010	106	1.9	0.5–6.7
	Great Bahama Bank–Orange Cay	Nov 2010	105	0	0.0–3.6
			788	**0.5**	**0.1–1.3**
Belize	Caye Caulker	Feb 2010	25	4.0	0.7–20.7
	Hol Chan Reserve	Feb 2010	2	0	0
	Glovers Atoll	Mar 2010	62	3.2	0.9–11.2
	Little Water Caye	Mar 2010	3	0	0
	Snake Bank	Feb 2010	8	0	0
	South Water Caye	Feb 2010	2	0	0
			102	**2.9**	**1.0–8.4**
Bermuda		Oct 2010	**102**	**0.0**	**0.0–3.7**
Cayman Islands	Grand Cayman	Oct 2010	102	0.0	0.0–3.7
	Little Cayman	Oct 2010	93	0.0	0.0–4.1
			195	**0.0**	**0.0–2.0**
Cuba	Guantanamo Bay	Mar 2011	48	4.2	1.1–14.3
	Guantanamo Bay	Nov 2011	31	9.7	3.2–25.7
			79	**6.3**	**2.7–14.1**
Curacao	North shore (various)	Nov 2010	31	0	0.0–11.8
	North shore (various)	Nov 2010	15	0	0.0–23.2
	North shore–Wakawa	Nov 2010	12	0	0.0–28.3
	South shore–Snakebaai	Nov 2010	2	0	0
	South shore–Caracasbaai	Nov 2010	9	0	0
	Perla Del Mar Restaurant	Nov 2010	51	0	0.0–7.3
			120	**0**	**0.0–0.32**
Dominican Republic	North coast	Aug 2010	99	3.0	1.0–8.6
	South coast	Aug 2010	111	5.4	2.5–11.4
			210	**4.3**	**2.3–8.0**
Honduras	Cayos Cochinos	Mar 2011	96	3.1	1.1–8.9
	Middle Bank	Mar 2011	51	0.0	0.0–7.3
	Banco Oneido	Mar 2011	50	2.0	0.3–10.8
			197	**2.0**	**0.8–5.1**
Mexico	Desterrada Key	Oct. 2010	72	5.6	2.1–13.6
	Desparecida	Oct 2010	27	3.7	0.6–19.3
	Isla Chica	Oct 2010	1	0	0
	Isla Perez	Oct 2010	1	0	0
			101	**4.9**	**2.1–11.2**
Martinique	La Trinite	Apr–Oct 2011	**101**	**0**	**0.0–3.8**
Nicaragua	Corn Island	Sep 2010	**99**	**0**	**0.0–3.8**
Panama	San Blas	Jul–Sep 2010	**99**	**1.0**	**0.2–5.6**
Puerto Rico	Mayaguez	Oct 2010–Apr 2011	**41**	**17**	**8.3–31.8**
St. Kitts	Dieppe Bay	Aug 2010	88	0	0.0–0.43
	Sandy Bay	Aug 2010	33	0	0.0–11.1
			121	**0**	**0.0–3.1**
United States	Lower Keys Middle Keys	Aug–Nov, Jan–Mar 2008–2009	**502**	**11.0**	**8.5–14.0**
Venezuela	Isla Margarita	Dec 2010	32	0.0	0.0–11.5
	Los Roques	Dec 2010	102	0.0	0.0–3.7
			134	**0.0**	**0.0–2.8**

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penalty of 0.1. Settings for the multiple alignment stage were an open gap penalty of 10.0 and an extend gap penalty of 0.05. Following ClustalW alignment, the resulting alignment was edited by hand. Sequences were examined for unique alleles using Collapse v.1.2. Measures of genetic distance and nucleotide diversity were performed using MEGA v.5.0 (Tamura et al. 2011).

To limit the possibility of counting PCR error or sequencing misreads as single nucleotide polymorphisms (SNPs), only those SNPs observed greater than 5 times at a single locus within the data set were counted in the analysis. We used 5 as the minimum number to establish an SNP as authentic because *Taq* error rates for PCR are estimated as 1.1×10^{-4} base substitutions per bp (Tindall & Kunkel 1988), meaning that more than 3 or 4 errors at any single location would be highly unlikely.

Rarefaction analysis

For cost-effectiveness, efficiency in sample processing, and quality control, we examined how the presence of alleles (allele richness) varied with the number of infected lobsters examined and the number of clones analyzed (4 vs. 8 bacterial clones sequenced per infected lobster). Rarefaction analysis on a preliminary data set was used to examine allele richness. Values for Cole's rarefaction were estimated using EstimateS 8.0 (Colwell 2006). In the first analysis, common and rare alleles from all of the clones (4 or 8 clones per infected lobster; see 'Molecular diagnostics and DNA sequencing') were used to examine allele richness. In the second analysis, we asked whether common alleles were represented in both the 4-clone and 8-clone data sets. Common alleles were defined as those present in 3 or more infected lobsters using 8 clones from each, or 2 or more lobsters using 4 clones from each. For each analysis, the number of clones (4 or 8) examined were run separately, and the presence of an allele was counted as 1 when 1 or more clones from an infected lobster had that allele.

RESULTS

PaV1 viral DNA was detected in adult *Panulirus argus* using PCR in 9 of the 16 countries surveyed (Table 1). In general, PaV1 was found in countries in the northern and western Caribbean Sea, but it was absent from the southeastern locations, particularly the islands of the Lesser Antilles. The highest prevalence of PaV1 was from Puerto Rico at 17% (out of a total sample size [n] = 41), followed by Florida at 11% (n = 502). We undertook extensive sampling around the Bahamas (n = 788) as part of an additional study (M. J. Butler et al. unpubl.). Collectively, the prevalence there was low (0.5%), and patchily distributed, with prevalence of 1 to 2% at 3 of 8 locations.

When all DNA sequences of the PaV1 amplicon were aligned, the total alignment length was 479 bp (primer sequences removed). Overall genetic distance (uncorrected p-values) between the PaV1 viral DNA sequences obtained from adult *Panulirus argus* was 0.0053, and mean nucleotide diversity was 0.0045. Whereas within-group diversity was low overall, the geographic location with the highest average within-group genetic distance was the Dominican Republic (0.0060), and the lowest was Honduras (0.0004) (Table 2). Genetic similarity between regions was analyzed using between-group genetic distance. The most genetically similar locations were Mexico and Honduras (0.0016) and Mexico and Belize (0.0017); the most genetically distant were the Bahamas and the Dominican Republic (0.0100) (Table 3).

PaV1 had a high allele richness, with 55 unique alleles identified from infected lobsters in which 8 clones were sequenced (n = 57 infected lobsters), and 61 unique alleles when 4 clones were sequenced (n = 90 infected lobsters) (Fig. 2). The 4-clone data set had a higher sample size; hence, more unique alleles were found therein. When common and rare alleles were analyzed together, allele richness did not show an asymptote in either the 4-clone or the 8-clone data; however, the 8-clone data had a steeper trajectory, indicating that somewhat more rare alleles were found when more clones were sequenced. The 4-clone data set identified 100% of the total number of alleles present. In the analysis of common alleles

Table 2. *Panulirus argus* virus 1 (PaV1) infecting *P. argus*. Average within-group genetic distance (uncorrected p-value) of DNA sequences of PaV1 obtained from adult spiny lobster

Location	Average genetic distance
Bahamas	0.0053
Belize	0.0027
Cuba	0.0039
Dominican Republic	0.0060
Honduras	0.0004
Florida Keys	0.0047
Mexico	0.0024
Panama	0.0048
Puerto Rico	0.0041

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Table 3. *Panulirus argus* virus 1 (PaV1) infecting *P. argus*. Average between-group genetic distance (uncorrected p-value) of DNA sequences of PaV1 obtained from adult spiny lobsters between sampling locations

	Puerto Rico	Panama	Mexico	Honduras	Dominican Republic	Cuba	Belize	Bahamas	Florida, USA
Puerto Rico	–								
Panama	0.0053	–							
Mexico	0.0040	0.0038	–						
Honduras	0.0033	0.0027	0.0016	–					
Dominican Republic	0.0064	0.0058	0.0047	0.0035	–				
Cuba	0.0054	0.0047	0.0035	0.0023	0.0054	–			
Belize	0.0048	0.0041	0.0029	0.0017	0.0048	0.0035	–		
Bahamas	0.0094	0.0093	0.0082	0.0071	0.0100	0.0091	0.0083	–	
Florida, USA	0.0057	0.0055	0.0043	0.0032	0.0063	0.0052	0.0045	0.0066	–

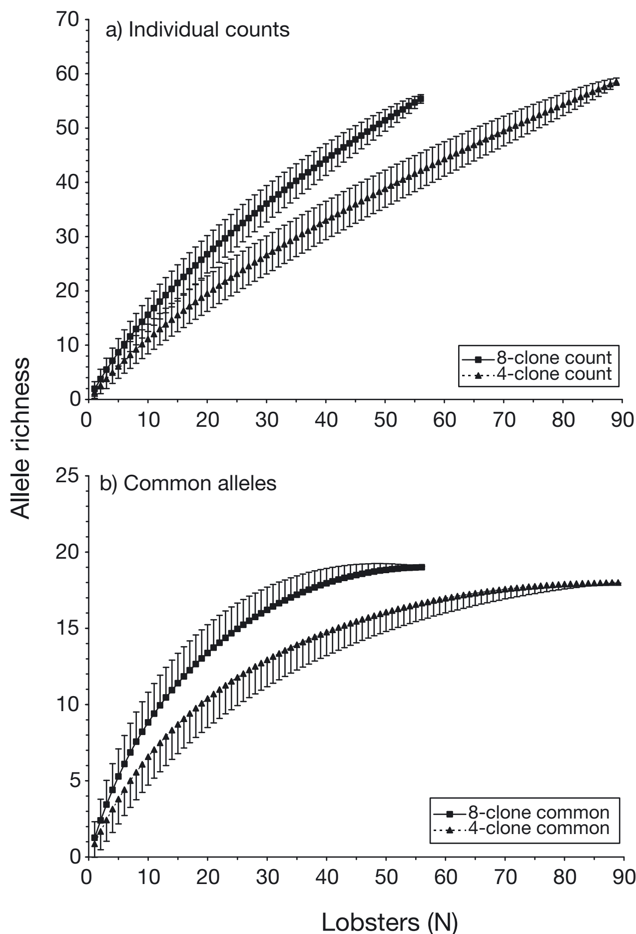

Fig. 2. *Panulirus argus* virus 1 (PaV1) infecting *P. argus*. Rarefaction analysis between 4-clone and 8-clone data sets for lobsters infected with PaV1. (a) Analysis of all alleles present in the data sets showing no approaching asymptote. (b) Analysis of common alleles indicating the number of lobsters needed to identify all of the common alleles (1 seen in more than 2 or 3 lobsters in 4- vs. 8-clone data sets, respectively) if sequencing 4 vs. 8 clones per lobster sample. Both sets are identifying the same common alleles

(n = 19), allele richness showed a distinct asymptote in both the 8-clone data set and the 4-clone data set, indicating that common alleles were representative in either set (Fig. 2b). The rarefaction analysis on common alleles indicated that DNA sequences of PaV1 from a total of 4 clones per lobster were representative of the population and that significant cost savings could be obtained using only 4 clones per lobster rather than 8 clones. Thus, 4 clones per infected lobster were used in the final analyses discussed below.

Many of the same polymorphisms observed previously (Moss et al. 2012) were found in other locations (Table 4), in addition to 19 new variable loci. Viral sequences contained 3 regions with microsatellites as well as many SNPs (Table 4). SNPs were recorded as any SNP that was observed ≥5 times in the data set. Thirty-eight unique sequences, or alleles, were new to the present study (Table 5). To examine the distribution of alleles and make inferences regarding sharing of alleles among regions, we combined data from Moss et al. (2012) with the present study (including viral alleles seen in postlarvae, EBJs, and juveniles from a viral mutation study). However, we limited the data set to only 4 clones per lobster as determined by rarefaction analysis (see previous paragraph). The most common allele was Allele 26 (frequency: 16%), followed by 12 (12%), 41 (10%), and 9 (9%); these 4 alleles were found in 47% of the clones analyzed. Single alleles constituted 29 of the 61 alleles observed. Representative sequences of all alleles have been deposited in GenBank (accession nos. JX987103 to JX987130).

Allele diversity was highest in Cuba (9 alleles), followed by the Dominican Republic (8 alleles). Diversity was lowest in Honduras, but the low prevalence limited the number of sequences obtained for PaV1 from that region. The greatest proportion of unique

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Table 4. *Panulirus argus* virus 1 (PaV1) infecting *P. argus*. Positions of PaV1 nucleotide variation. Superscripts next to microsatellite sequences indicate number of repeats observed. For single nucleotide polymorphism (SNP) notation, the most common base observed is listed first, followed by the observed base change. **Bold text** indicates polymorphisms found previously (Moss et al. 2012)

Base position	Variation
10–33	**(CGA)$^{4-8}$**
24	A/G
35–36	AG/GA
70	**C/T**
87	**A/C**
78–107	**(ACG)$^{6-10}$**
130	G/A
113	A/C
115	A/C
119	T/C
120	C/T
121–132	GAC GAC GAC GCC deletion
125	**A/C**
129–143	**(CCG)$^{2-4}$**
131	C/A
134	C/A
185	A/G
236	**T/C**
245	G/A
246	C/T
251	**A/G**
261	C/T
300	G/A
301	G/T
306	**A/G**
333	A/G
363	C/T
375	**C/T**
399	**G/A**
411	**T/C**
416	A/G
438	**C/T**

alleles occurred in adult lobsters from the Bahamas (80% of alleles), Florida (57%), the Dominican Republic (50%), and Cuba (44%), with 4 unique alleles each. Seven alleles were shared between 2 different locations (Fig. 3). One allele found in Florida was shared between the Dominican Republic and Puerto Rico (sharing between 3 locations), and 3 alleles found in Florida were shared with 3 additional locations (Allele 12: Dominican Republic, Mexico, and Panama; Allele 26: Belize, Dominican Republic, and Mexico; Allele 41: Cuba, Dominican Republic, and Honduras sharing between 4 locations).

In some cases, individual spiny lobsters were infected with multiple alleles of PaV1, which is suggestive of multiple infections (Table 6). In 13 cases, a single lobster contained 2 viral alleles, and in 3 instances, a single lobster contained 3 different viral alleles.

DISCUSSION

PaV1 is undetectable in adult lobsters via visual inspection, but genetic analysis demonstrates that PaV1 is widespread in adult spiny lobsters in central and northern portions of the Caribbean Sea, has a low prevalence in the southwestern Caribbean, and is absent from the 476 lobsters we sampled at the 4 southeastern Caribbean locations. Adult lobsters from Puerto Rico had the highest prevalence of PaV1 (17%). Our results extend the known range of the virus in Belize, Cuba, Florida, Mexico, and St. Croix (US Virgin Islands) to now include Puerto Rico, the Dominican Republic, the Bahamas, Honduras, and Panama. Overall, the virus had a low prevalence (0.5%) in adult lobsters from the Bahamas and was present at only 3 of 8 sites that we surveyed, indicating that it is not widely distributed there. The prevalence of PaV1 in adult lobsters from Cuba was 6.3%, higher than that reported previously (Cruz Quintana et al. 2011: 1.5% average from 4 locations). A possible explanation may be the difference in sampling locations, as the lobsters in the present study were collected from Guantanamo Bay, whereas the previous study examined lobsters from the Gulf of Batabanó. Our finding of 3.2 to 4.0% (mean: 2.0%) prevalence off Belize is in sharp contrast to the 50% prevalence reported from frozen lobster tails exported from Belize (Huchin-Mian et al. 2009). That study did not state where the lobsters were caught in Belize, but the prevalence was far higher than that reported in the present study.

We did not find PaV1 in adult lobsters from the southeastern Caribbean locations (Martinique, St. Kitts, Venezuela, and Curaçao), nor from Bermuda. At least 100 lobsters were sampled from each of these locations. We cannot rule out the presence of the virus from these locations, as hundreds of animals would be required from each location to verify prevalence levels below 1.0% (Gregory & Blackburn 1991, Jovani & Tella 2006), but if present, it has a very low prevalence in those areas. However, we speculate that the net movement of currents in the Caribbean basin from the southeast to the northwest (http://oceancurrents.rsmas.miami.edu/caribbean/caribbean.html) may minimize the spread of the virus to 'upstream' locations in the eastern Caribbean.

The genetic diversity of PaV1 in the Caribbean Sea was high, with 61 unique viral alleles from 9 locations. Not only was the diversity high, but all locations except Honduras possessed alleles unique to those locations. For example, 2 of the 3 alleles observed in Panama were unique (66%), only occurring

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Table 5. Distribution of *Panulirus argus* virus 1 (PaV1) alleles in Florida, USA, and the Caribbean Sea. Alleles in **bold** are those previously reported (Moss et al. 2012). Additional single nucleotide polymorphisms (SNPs) are reported as new alleles. Only 4 clones per lobster were used in this analysis. PLs: postlarvae; EBJs: early benthic juveniles (<15 mm carapace length); FL: Florida; BA: Bahamas; BZ: Belize; CU: Cuba; DR: Dominican Republic; HO: Honduras; MX: Mexico; PN: Panama; PR; Puerto Rico

Allele	FL PLs[a]	FL EBJs[a]	FL juveniles[a]	FL adults	BA	BZ	CU	DR	HO	MX	PN	PR
1		3								4		
2		1										
3		22										
4		5					4					
5		1										
6		4										
7		1										
8		1										
9		34										
10	4	7				1						
11		1										
12	22	14						2		2	2	
13		1										
14		2										
15		1										
16	1		1					1				1
17	1											
18	1											
19	1											
20	1											
21	8											
22	1											1
23		1										
24		3										
25		1										
26		3	21	11		10		2		11		
27			2									
28		1										
28		8			4							
30		1										15
31		3										
32		1										
33												3
34												1
35												1
36												4
37											1	
38											1	
39										1		
40										4		
41				3			3	20	12			
42							1	1				
43								4				
44								1				
45								1				
46							1					
47							3					
48						3	4					
49				1			1					
50							2					
51							1					
52						1						
53						1						
54					6							
55					4							
56					1							
57					1							
58				4								
59				1								
60				4								
61				4								
Total	9	24	3	7	5	5	9	8	1	5	3	7
Unique	5	16	1	4	4	2	4	4	0	2	2	3

[a]Samples from Moss et al. (2012)

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in Panama, whereas 4 of the 5 alleles found in the Bahamas were unique (80%). This suggests that although many alleles are shared between sites, an indication of high viral genetic connectivity, some alleles were endemic to certain locations and thus may be useful indicators for defining host populations. Although the genetic distance between PaV1 from Mexico and Honduras was most similar (uncorrected p-distance: 0.0016), these 2 regions did not share alleles for PaV1. However, our samples from Mexico were obtained from Alacranes Reef in the Gulf of Mexico to the northwest of the Yucatan Peninsula, an area quite distinct hydrographically from Mesoamerica. So although the PaV1 samples from Belize were genetically similar to Mexico (0.0029 relatedness), they were more closely aligned with Honduras (0.0017); samples from Belize and Honduras only shared 1 viral allele.

Based on our existing DNA sequences, the most genetically distinct population of PaV1 was in the Bahamas. Samples of the virus from the Bahamas had relatively high between-group genetic distances compared to PaV1 in other regions. Bahamian PaV1 DNA sequences were genetically most different from the Dominican Republic (uncorrected p-distance: 0.0100) and shared no viral alleles. Given the close proximity of the Bahamas to the Dominican Republic, this may seem surprising. However, hydrographic modeling of larval lobster dispersal indicates that the complex bathymetry of the Bahamas constrains lobster population connectivity (Lipcius et al. 1997, Butler et al. 2011), which is also consistent with hydrodynamic modeling and empirical data on population connectivity of other species in the Bahamas (Cowen et al. 2006, Galindo et al. 2006). Indeed, 80% of the PaV1 alleles from Bahamian lobster samples were unique and shared only 1 viral allele with Florida, indicative of a significant barrier to viral gene flow that largely isolates the Bahamas.

Table 6. *Panulirus argus* virus 1 (PaV1) infecting *P. argus*. Frequency of multiple infections in spiny lobster. N = number of PaV1-positive samples sequenced from the specific location

Location	N	2 alleles	3 alleles
Bahamas	4	1	1
Belize	4	2	0
Cuba	5	2	1
Dominican Republic	8	2	0
Florida, USA	7	4	0
Honduras	3	0	0
Mexico	5	1	0
Puerto Rico	7	0	1
Panama	1	1	0

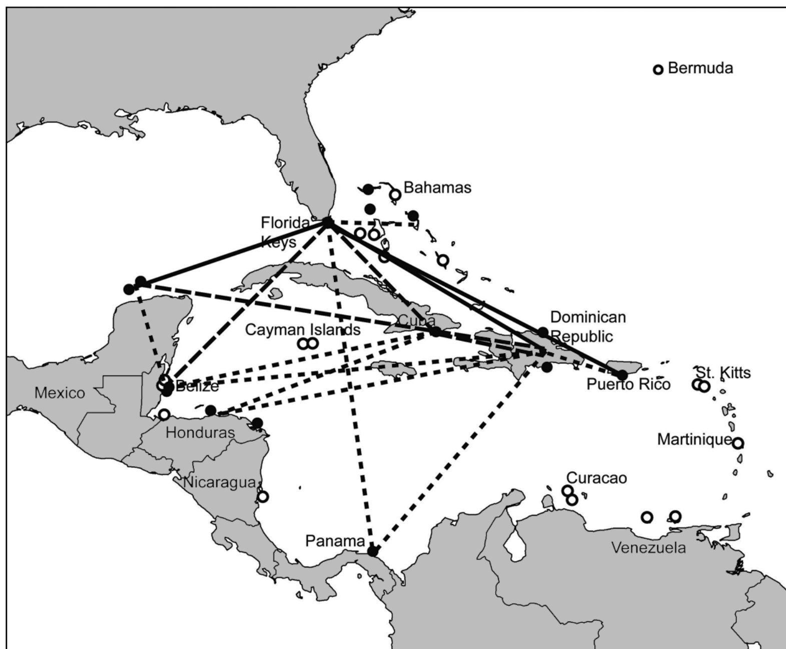

Fig. 3. *Panulirus argus*. Sampling locations (●), and genetic connectivity between populations with *Panulirus argus* virus 1 (PaV1). Locations where lobsters were sampled but where PaV1 was absent are indicated (○). Genetic connectivity: 3 (—), 2 (– –), or 1 (- - -) viral allele(s) shared

The overall genetic distance among viral DNA sequences from adult spiny lobsters was 0.0053. With the exception of the viral alleles from the Bahamas, our phylogenetic analyses of viral alleles, using both neighbor-joining and parsimony analyses, reveals little population structure based on geographic location (J. Moss unpubl. data). This is comparable to other reports that have discerned little structure in the population genetics of *Panulirus argus*, using allozymes (Menzies 1980), restriction fragment length polymorphisms in mitochondrial genes (mtDNA RFLP) (Silberman et al. 1994), sequencing of the mitochondrial 16S ribosomal gene (Sarver et al. 1998), and segments of the mitochondrial cytochrome *c* oxidase subunit I gene (Naro-Maciel et al. 2011). However, a recent preliminary analysis of lobster genetic structure in Mesoamerica based on neutral genetic microsatellite analyses suggests that subregional population structure

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may exist in that region (Truelove et al. 2012). The long planktonic larval duration (PLD) of *P. argus* (5 to 7 mo; Goldstein et al. 2008), and thus potential for long distance dispersal (Lyons 1980, Silberman et al. 1994, Briones-Fourzán et al. 2008, Naro-Maciel et al. 2011), provide a mechanism for what is generally considered a panmictic population. However, when biophysical models incorporate larval behavior with complex current models, the results indicate that *P. argus* larvae are often entrained within a few hundred kilometers of where they were spawned (Butler et al. 2011). This is particularly so in environments with retentive oceanographic circulation patterns, such as the Bahamas, where larvae are most likely to be retained, possibly facilitating the rise of unique alleles for both the host and the virus. Still, biophysical models predict that nearly 10% of *P. argus* larvae disperse >1000 km from many Caribbean spawning sites, highlighting the disparity between demographically relevant and genetically relevant population connectivity (Butler et al. 2011).

Given that PaV1 is widespread in spiny lobster populations throughout the northwestern Caribbean Sea, and viral alleles are often shared between distant locations, there is a high degree of disease connectivity in a large portion of the Caribbean. Yet, laboratory transmission of PaV1 among lobsters and in cell culture indicates that PaV1 is only viable for a few days in seawater (Butler et al. 2008, M. J. Butler et al. unpubl. data). Moreover, the depth of the seafloor separating lobster populations and the cold temperatures on the bottom of those deep channels are a barrier to deep sea movement of tropical spiny lobsters. So how can viral alleles be shared over such large distances in the Caribbean? Newly arriving lobster postlarvae in the Florida Keys have been diagnosed with PaV1 (Moss et al. 2012), and we have preliminary evidence for maternal transmission to the embryos (vertical transmission). Thus, larval transport is a potential means for dispersal of the virus through the Caribbean.

The viral diversity is highest within the Florida Keys, and this may either be a real phenomenon as currents from around the Caribbean move through the Florida Straits, or it could be an artifact of our sequencing a large number of PaV1 samples from the Florida Keys compared to other regions. Moreover, the large number of PaV1 alleles described (n = 61), their extensive geographic distribution, and the presence of unique alleles at most of the regions sampled is highly suggestive that the virus has been present in the Caribbean for a long time. That is, it is unlikely that PaV1 is a newly emergent pathogen.

Given that EBJs are highly susceptible to PaV1, and once infected, experience very high rates of mortality (Butler et al. 2008, Behringer et al. 2011), it is clear that PaV1 may have a significant effect on lobster fisheries. Caribbean-wide landings of spiny lobsters peaked during the period 1987–1997 but have since decreased by 55% in the 2000s, with environmental degradation and overexploitation generally blamed for the decline (Ehrhardt et al. 2010). Although PaV1 was first discovered in 1999 (Shields & Behringer 2004), we do not know when it emerged nor do we know its primary mode(s) of dispersal. Yet, the most dramatic declines in spiny lobster landings in the past decade have been in Cuba (60% mean landings decrease, PaV1 prevalence of 6.3% in adults) and Florida (50% mean landings decrease, PaV1 prevalence of 11% in adults), 2 locations where PaV1 had a relatively high prevalence. Landings in the Nicaragua–Honduras region have declined by less than 20% (Ehrhardt et al. 2010), and in the present study, PaV1 had a low prevalence in Honduras (2%) and was absent from our 1 sample location in Nicaragua. In Florida, the prevalence of PaV1 has been reported as high as 11% in adult lobsters, whereas in some localized 'hot spots', prevalence in juveniles has reached as high as 60% (Behringer et al. 2011). Likewise, in Cuba, PaV1 prevalence in adults may be low (0.83%) but has reached over 44% in juveniles in some locations (Cruz Quintana et al. 2011). Therefore, our estimates of PaV1 prevalence in *Panulirus argus*, based on sampling of adult lobsters alone, underestimates its prevalence in lobster populations where PaV1 prevalence is undoubtedly higher in juveniles, which are more difficult to sample.

The potential for damage by PaV1 to one of the Caribbean's most iconic and economically valuable fisheries underscores the need for management that considers the very real impact that PaV1 may have on lobster stocks. Estimates of natural mortality rates, especially for EBJs and juveniles, may be significantly higher if PaV1 is present. For example, based on mean prevalence of visible infections in juvenile lobsters from 2000 to 2010 (Behringer et al. 2011), we estimate that at least 24% of the settlers in Florida never recruit to fishery size due to mortality from PaV1 infection. Accordingly, we suggest that disease surveys be undertaken in adult and small juvenile stages in all nations with significant spiny lobster fisheries, and particularly those where the prevalence exceeds 1% in adult lobsters.

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