Genetic diversity of the Caribbean spiny lobster virus, *Panulirus argus* virus 1 (PaV1), and the discovery of PaV1 in lobster postlarvae

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ABSTRACT: *Panulirus argus* virus 1 (PaV1) is an unclassified, pathogenic virus first discovered in Caribbean spiny lobsters *Panulirus argus* from the Florida Keys, USA, and has since been confirmed in lobsters in other parts of the Caribbean. Several studies have examined the prevalence, pathogenicity, and population dynamics of the pathogen in the lobster host, but little is known about the genetic diversity of the virus. We analyzed a fragment of viral DNA from lobsters collected in the Florida Keys, which provided the first insight into the genetic diversity of PaV1. The variation in viral sequences, both within and between individual lobsters, indicates that lobsters can be infected with multiple viral strains. Laboratory experiments in which uninfected lobsters were serially exposed to lobsters inoculated with PaV1 implied that rapid mutation of strains is unlikely and suggest that there may be endemic strains of PaV1. In addition, we discovered PaV1 in *P. argus* postlarvae (pueruli), suggesting that the virus may disperse through the Caribbean within the long-lived (5 to 7 mo) planktonic phyllosoma larvae — a novel mechanism for dispersal of viruses in the sea.

KEY WORDS: Genetic variation · Pueruli · Vertical transmission

INTRODUCTION

Panulirus argus virus 1 (PaV1) is a large, icosahedral virus that is pathogenic in the Caribbean spiny lobster *Panulirus argus*. Although as yet unclassified, PaV1 was first described in the Florida Keys, USA, in 1999 (Shields & Behringer 2004). Infections have since 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); thus, it is probably widespread in the Caribbean. The occurrence of disease has an ontogenetic component, with the highest prevalence and heaviest infections in early benthic juveniles (EBJs), and a re-

duction in prevalence and intensity among the larger juveniles and adults (Shields & Behringer 2004, Butler et al. 2008, Lozano-Álvarez et al. 2008). PaV1 prevalence in juvenile lobsters in Florida has ranged from 2 to 8%, with some locations exceeding 40% (Behringer et al. 2011). Although adult lobsters are generally asymptomatic, they can be infected by PaV1, as has been determined through polymerase chain reaction (PCR) screening of adults in Florida (prevalence: 11%) (Behringer et al. 2012), Belize (prevalence: 50%) (Huchin Mian et al. 2009), and Cuba (prevalence: 4.48%) (Cruz Quintana et al. 2011).

Heavily infected lobsters are typically lethargic, cease grooming and foraging, and have lower hemo-

lymph serum protein values (Behringer et al. 2008). Juvenile and adult Caribbean spiny lobsters are usually social but this behavior is affected by infection with PaV1; in addition uninfected lobsters avoid diseased lobsters in the wild (Behringer et al. 2006).

Although the chalky-white appearance of infected hemolymph, viewed through the juncture of the abdomen and ventral carapace, has been used to diagnose PaV1 infections, it only reveals the most severely diseased individuals. More accurate diagnostic methods for PaV1 include histological analysis of preserved tissue sections, fluorescent *in situ* hybridization (Li et al. 2006), cell culture methods (Li & Shields 2007), and a PCR assay (Montgomery-Fullerton et al. 2007). The primers for the PCR assay amplify an approximately 500 bp section of a 982 bp viral fragment (GenBank accession no. EF206313) that appears to be an open reading frame (ORF) with no known homologs. Molecular detection of viral DNA using a PCR-based assay has been used to diagnose PaV1 in *Panulirus argus* from Florida (Behringer et al. 2012), Mexico (Huchin-Mian et al. 2008), Belize (Huchin-Mian et al. 2009), and Cuba (Cruz Quintana et al. 2011). For routine diagnosis of PaV1, we optimized the previously published PCR assay (Montgomery-Fullerton et al. 2007). During the optimization and validation of the modified PCR assay, sequencing of PaV1 isolates revealed inter-sequence nucleotide variation that had not been previously reported.

The objectives of the present study were to examine the sequence variation in PaV1 viral DNA from EBJ lobsters collected in the Florida Keys and postlarvae recruiting from offshore, and compare it with virus samples from juvenile lobsters used in laboratory disease challenges.

MATERIALS AND METHODS

Postlarvae collection

Transparent postlarvae (i.e. pueruli) of *Panulirus argus* were collected monthly using Witham-type artificial collectors (as in Butler & Herrnkind 1991) moored at 2 locations in the nearshore Atlantic Ocean, within 0.5 km of Long Key, FL, USA (Fig. 1) from February to July 2007. Postlarvae arriving from the oceanic planktonic environment can be caught on these collectors around the time of the new moon (Acosta et al. 1997). The transparent postlarvae obtained from collectors were estimated to have been in the coastal waters for a few days at most, as they
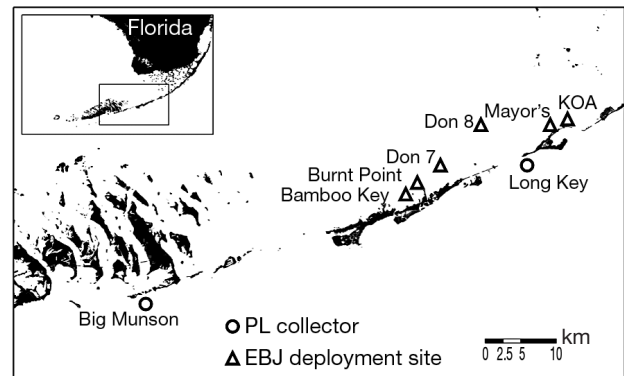

Fig. 1. Field sites in the Florida Keys, USA (24.82° N, 80.82° W). EBJ: early benthic juveniles, PL: postlarvae

rapidly develop pigments upon settling in near-shore nursery habitats. After collection, postlarvae were frozen whole at −20°C or −80°C for PCR diagnostics.

EBJ sentinel study

EBJs were deployed as sentinels for PaV1 at 6 field sites in the Florida Keys in 2007. The sentinels were screened using PCR for the presence of PaV1 DNA only after deployment. The original purpose of the sentinel study was to determine the incidence of PaV1 in recently settled EBJs and young juveniles.

For the sentinel study, postlarvae were collected 7 d following the new moon from May to July 2007 from Witham-type collectors deployed oceanside of Long Key and Big Munson Key (Fig. 1) and held in re-circulating aquaria with UV-sterilized water for <1 wk until they metamorphosed into EBJs. EBJs were fed thawed brine shrimp and finely minced squid every other day. On the day of deployment, 1 or 3 EBJs were placed inside 0.25 m^2 Vexar mesh cages (total of 30 to 50 EBJs per site) along with approximately 4 l of their preferred settlement substrate, the red macroalgae *Laurencia* spp. (Herrnkind & Butler 1986). The cages were then attached to the seafloor at 1.5 to 3 m depth at 6 hardbottom lobster nursery sites in the middle Florida Keys (Fig. 1). The cages were deployed for 2 wk and then retrieved, and the remaining EBJs recovered and dissected; the tails were frozen individually in vials for later PCR diagnostics and the thoraxes were fixed and stored individually in 10% neutral buffered formalin for histology. For controls, we held 6 to 9 EBJs in the laboratory each month in individual 10 l aquaria with UV-sterilized flow-through seawater for the same time

period as those deployed in the field. Control EBJs were processed for analysis as described for EBJs from the field.

Viral mutation study

Six hemolymph samples from a previous 'time-to-transmission' experiment (see Behringer et al. 2006) were used to study the molecular variation of PaV1 in a laboratory setting. Specifically, we used the 6 samples to test whether the genetic variation we observed in viral isolates from individual lobsters was due to a high viral mutation rate after a single infection episode or whether viral variation within a lobster reflected multiple viral infections. In summary, 12 naive juvenile lobsters (20 to 35 mm carapace length) were inoculated with 50 µl of PaV1-infected hemolymph from a single donor and held individually in 10 l aquaria supplied with UV-sterilized flow-through seawater for 2 wk; 4 lobsters inoculated with hemolymph from a visibly uninfected donor collected from the wild and treated in the same manner served as controls. Hemolymph from the infected and uninfected donors was saved in 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) and frozen at −80°C. At 2 wk intervals over 8 wk (4 'trials' total), a single naive lobster was held in each aquarium with one of the inoculated lobsters for 2 wk (Fig. 2). Thus, 12 naive lobsters occupied separate aquaria, each with a lobster inoculated with PaV1-infected hemolymph, while 4 other naive lobsters were held each with a control lobster inoculated with hemolymph from a naive lobster. After 2 wk, 16 more naive lobsters replaced those in aquaria in the previous exposure trial (12 with PaV1-infected lobsters, 4 with naive lobsters), and this procedure was repeated every 2 wk for 8 wk.

At the end of each 2 wk exposure, the exposed lobsters were removed from the experimental aquaria and held in separate individual aquaria for approximately 80 d to allow for overt development of PaV1 infections. Initially, disease status during the experiment was assessed by examining the hemolymph of each lobster through the juncture of the carapace and abdomen. White hemolymph was indicative of viral infection. This was later confirmed in our laboratory using PCR. At the end of the holding period, all surviving animals were necropsied. Tissue samples from all lobsters, including the original PaV1 donor, the inoculated lobsters, and the exposed lobsters,

were histologically processed. Hemolymph samples were drawn from all animals and the hemolymph frozen in anticoagulant at −20°C or −80°C prior to molecular diagnosis. The 6 hemolymph samples used to assess viral mutation included that from the donor and the 5 individuals that acquired the virus through exposure as seen in Behringer et al.'s (2006) Trial 4 (see 'Results — Viral mutation study').

DNA extractions

DNA extractions were performed on 100 to 150 µl of lobster hemolymph, or on ~100 to 150 mg of lobster muscle tissue taken from the anterior portion of the tail of either postlarvae or EBJs. All extractions were carried out using a Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol. Lobster genomic DNA was eluted in 150 µl (hemolymph samples) or 200 µl (tissue samples) of elution buffer and stored at 4°C until assayed.

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

The diagnosis of PaV1 was done using the PCR primers published previously (Montgomery-Fuller-
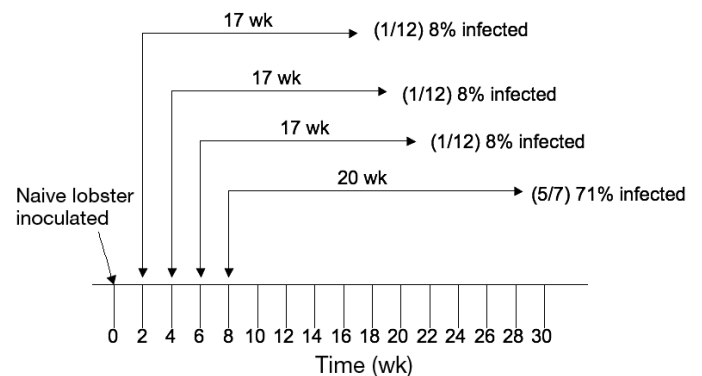

Fig. 2. *Panulirus argus* virus 1 (PaV1) infecting *Panulirus argus.* Experimental design of the PaV1 viral mutation study (redrawn from Behringer et al. 2006). At Time 0, 12 lobsters were inoculated with hemolymph from a single donor infected with PaV1. Trial 1: 2 wk after inoculation, 12 naive lobsters were exposed to the inoculated/infected lobsters for 2 wk. They were removed and held separately for 17 wk. Trials 2, 3, and 4, all at 2 wk intervals: more naive lobsters were exposed to the inoculated lobsters for 2 wk, removed, and maintained separately for 17 or 20 wk. The number and percentage of PaV1-positive (infected) lobsters after 17 or 20 wk post exposure to inoculated lobsters are indicated

ton et al. 2007), with modifications to reagent concentrations, thermocycling parameters, and reaction volume. 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 forward primer, 0.5 µM reverse primer, and 1.0 unit *Taq* polymerase. 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 (ddH$_2$O) plus PCR reagents in the above concentrations (no-DNA control) was included. 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, and 72°C for 1 min, all followed by a final elongation step at 72°C for 10 min. Aliquots of 10 µl of PCR 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 Fluor-Chem® imaging system. We determined the sensitivity of the modified PCR protocol to be 0.02 fg µl^{-1} viral DNA or 0.05 fg of viral DNA per PCR reaction using plasmid DNA of the viral sequence.

Cloning and sequencing

To assess the genetic variability of PaV1, a subset of PCR-positive samples were cloned and sequenced from naturally infected and laboratory-infected lobsters (see Table 1). 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 volume 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 one-eighth the concentration of Big Dye recommended by the manufacturer's protocols. Sequencing reaction products were re-suspended in 20 µl of Hi-Di formamide (Applied Biosystems) and 10 µl of each product were elec-

trophoretically separated on an ABI 3130 Prism Genetic Analyzer.

Vector trimming and sequence editing were performed using CodonCode Aligner v. 3.0.3. Sequences were aligned using MacVector v. 8.1.2 and compared using Collapse v. 1.2 and Arlequin v. 2.0. TCS 1.21 was used to create a minimum spanning network of the observed viral alleles at the sequenced locus. FgenesV (http://linux1.softberry.com/), a trained pattern/Markov chain-based viral gene prediction program, was used to translate the sequenced region and to identify ORFs or possible viral genes.

RESULTS

PaV1 detection by PCR

Postlarvae

PaV1 DNA was detected in transparent postlarvae obtained from Witham collectors near Long Key in 3 of the 4 months sampled in 2007 (Table 1). Prevalence ranged from 30% in February to 0% in April. Transparent postlarvae arriving from offshore become pigmented within a day or two of settlement, thus our PCR results indicate that the postlarvae arrived from offshore with PaV1 infections.

EBJs

PaV1 DNA was detected in EBJs deployed as sentinels at all 6 field sites in the Florida Keys (Table 1). Prevalence of the virus ranged from 3% in the Bamboo Key samples to 71% in the Don 8 samples (see Fig. 1 for study sites), averaging 48%. However, the sentinel study was flawed because PaV1 was prevalent in sentinel animals and controls (mean: 40%; 2-sample *t*-test: $t = 0.415$, df = 7, p = 0.691). Again, this indicates that a substantial portion of the postlarvae obtained from Witham plankton collectors and then deployed as sentinels or held individually in laboratory controls with UV-treated seawater were already infected with PaV1 when they arrived inshore. We could not determine whether EBJs caged as sentinels for 2 wk acquired additional infections.

Viral mutation study

Hemolymph samples were only analyzed by PCR in infected individuals (as determined histologically

Table 1. *Panulirus argus* virus 1 (PaV1) infecting *Panulirus argus*. Postlarvae and early benthic juveniles (EBJs) were analyzed for the presence and prevalence (prev.) of PaV1 by PCR, and examined for viral diversity

Site	Collection month	No. ind. collected	No. PaV1-positive by PCR	PaV1 prev. (%)	No. ind. sequenced	No. clones sequenced	No. alleles observed	Nucleotide diversity (π)
Transparent postlarvae obtained from Witham collectors								
Long Key	Feb	20	6	30	6	55	4	0.001854
Long Key	Mar	20	1	5	–	–	–	–
Long Key	Apr	20	0	0	–	–	–	–
Long Key	May	20	4	20	4	38	5	0.019362
EBJs deployed as sentinels on nursery sites								
Burnt Point	May	30	18	60	4	38	4	0.002346
Bamboo Key	May	30	1	3	1	11	2	0.002791
Don 7	Jun	40	28	70	4	32	4	0.007163
Don 8	Jun	51	36	71	4	34	4	0.010404
Mayor's	Jul	35	15	43	4	33	8	0.014918
KOA	Jul	37	16	43	4	37	5	0.011654
EBJs held in UV-treated seawater as sentinel controls								
Lab controls	May	7	0	0	–	–	–	–
Lab controls	Jun	8	5	63	5	40	5	0.007296
Lab controls	Jul	7	4	57	4	40	3	0.014228

by Behringer et al. 2006) from the last exposure period in the time-to-transmission study (Trial 4). That is, naive lobsters were examined after their exposure for 2 wk to lobsters inoculated 8 wk earlier with infected hemolymph. All individuals determined to have PaV1 based on histology in Behringer et al.'s (2006) Trial 4 (n = 5) were also positive for PaV1 DNA by PCR. The aliquot of frozen hemolymph from the original donor source of PaV1 for the experiment was also positive for PaV1 by PCR.

Genetic analysis

EBJs and postlarvae

Of the EBJ samples that were PCR-positive, 4 lobsters from each site, except Bamboo Key (only 1 positive sample), were cloned, and at least 8 clones from each site were bi-directionally sequenced. All of the EBJs used as controls for this study that were PCR-positive were also sequenced. Of the postlarvae that were PCR-positive from Long Key, viral DNA from all but one individual was cloned and at least 8 clones were sequenced (Table 1). Sequenced fragments of PaV1 ranged from 452 to 465 bp in length (primer sequences removed). These sequences corresponded to bases 65 through 523/536 of the PaV1 sequence in GenBank (accession no. EF206313). When the software program FgenesV was used to translate the sequenced product, bases 145 to 465 (alignment posi-

tion) could be translated into a possible viral gene of 105 amino acids in length.

Within the ~500 bp fragment of PaV1, there were 3 regions containing microsatellites and 10 major single nucleotide polymorphisms (SNPs) (Table 2). To limit the possibility of counting PCR error or sequencing misreads as SNPs, only those SNPs observed >5 times at a single locus within the data set were used in the analysis. We used 5 as the minimum number to establish a SNP as authentic because *Taq* error rates for PCR are estimated as 1.1×10^{-4} base substitutions per base pair (Tindall & Kunkel 1988), meaning that more than 3 or 4 errors at any single location would be highly unlikely. Of all the SNPs examined, only 3 appear to cause an amino-acid change. Those amino-

Table 2. *Panulirus argus* virus 1 (PaV1). Positions of PaV1 nucleotide variations. **Bold**: microsatellite sequences, with superscripts indicating the number of repeats observed. Nonbold: single nucleotide polymorphism (SNP), with the most common base observed listed first, followed by the observed base change. An asterisk (*) indicates those SNPs that caused an amino-acid change in the putative open reading frame

Base position	Variation	Base position	Variation
10–33	**(CGA)$^{4-8}$**	249	A/G*
70	C/T	304	A/G*
78–108	**(ACG)$^{6-10}$**	373	C/T
87	A/C	397	G/A
123	A/C	409	T/C
129–140	**(CCG)$^{2-4}$**	436	C/T
234	T/C*		

acid changes were at base positions 234 (isoleucine/valine), 249 (cysteine/arginine), and 304 (aspartic acid/glycine) (5′–3′ notation with positions based on an alignment of all sequences with primers removed). Three microsatellites were observed; all were trinucleotide repeats (Table 2). Nucleotide diversity, calculated in Arlequin, ranged from the lowest in the postlarvae from the Long Key samples of February (0.001854) to the highest observed from the Long Key postlarval samples of May (0.019362) (Table 1).

Viral mutation study

There was little PaV1 sequence variation observed in the lobster inoculated with the donor PaV1 hemolymph at the start of the time-to-transmission experiment. Two alleles were present in the 8 clones analyzed from the PaV1 donor. One allele was found in 7 clones, while the other was observed only once. The dominant allele was compared to the 8 clones sequenced from the 5 lobsters infected with the virus in Trial 4 (40 sequences in total). Only 1 clone of the 40 clones was different from the dominant allele from the donor. The unique allele was not the rare allele seen in the donor; it either represents a mutation in the virus or a rare allele not initially identified when sequencing clones from the donor lobster. Note also that the naive lobsters exposed to the inoculated lobsters may have been naturally exposed to the virus; they were not pre-screened by PCR because the assay had not yet been developed, so it is possible that a small fraction of those allocated into the 4 trials could have already carried undetected low-level infections.

When the sequence data from the sentinel EBJs, postlarvae, and mutation experiment samples were combined, 33 alleles were identified (Table 3). By alleles, we mean unique sequences where all microsatellite and SNP regions were included. A minimum spanning network shows the genetic distance and relationships among alleles (Fig. 3). Specifically, this figure indicates the nucleotide differences between alleles, whether that is due to a microsatellite, or a SNP, or a combination of both. Representative sequences for each allele have been deposited in GenBank (accession nos. JN786952 to JN786984). Many of these alleles were found only once (i.e. in a single clone within a single individual lobster). When the singletons were removed from the data set, a total of 15 alleles remained. The most prevalent allele was 14, followed by alleles 9 and 3 (Table 3). The greatest number of viral alleles from a single group of lobsters were 8 from the EBJs deployed at the Mayor's site in

July. Most lobsters contained 1 or 2 viral alleles, although 6 individuals (5 EBJs and 1 postlarvae) had 3 alleles at the PaV1 locus; these combined data may be indicative of multiple infections of different viral strains.

Table 3. *Panulirus argus* virus 1 (PaV1) infecting *Panulirus argus*. Allele frequencies for PaV1 observed within each lobster sample. Bamboo Key, Burnt Point, Don 7, Don 8, KOA, and Mayor's: virus samples from early benthic juveniles (EBJs). LK: virus samples from postlarvae collected at Long Key. Lab controls: EBJs held under UV and served as controls for the sentinel study. VM: viral samples from juvenile lobsters sequenced as part of the viral mutation study

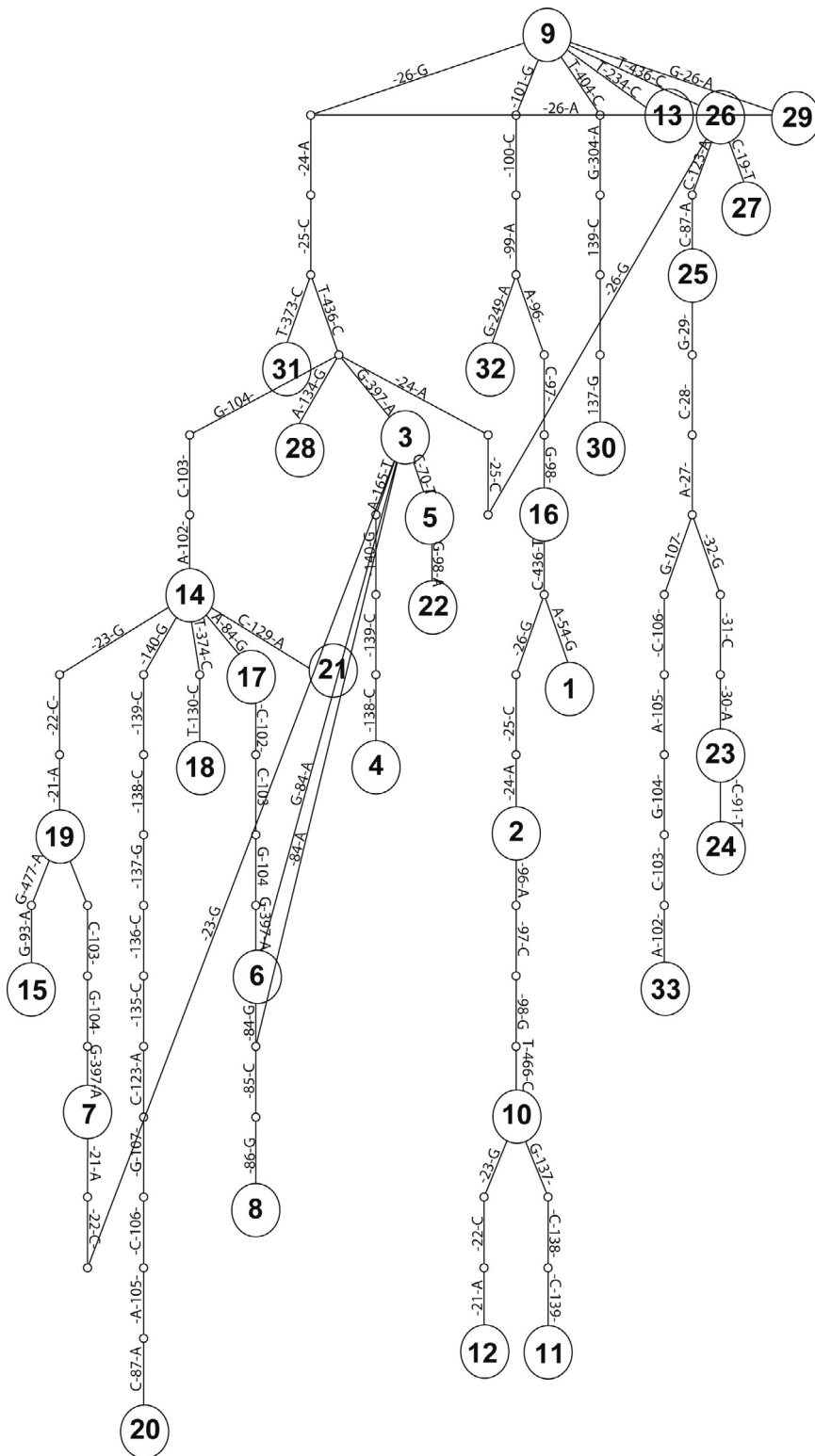| Allele	Bamboo Key	Burnt Point	Don 7	Don 8	KOA	Mayor's	LK Feb	LK May	Lab controls Jun	Lab controls Jul	VM study
1	10										
2	1										
3		34	14	4							
4		2			1				8		
5		1				7					
6		1									
7			1								
8			1								
9			16	16	9	3			29		
10				14				8			
11				1							
12				1				1			
13				1							
14					23	8	50	9		8	
15					2						
16					1						
17							1				
18							1				
19							3				
20								17			
21								1			
22						1					
23						7					
24						1					
25						1					
26						5					47
27											1
28											1
29									1		
30									1		
31										16	
32									1		
33										8	
Sum	2	4	4	6	5	8	4	5	5	3	3

Fig. 3. Minimum-spanning network of viral alleles from *Panulirus argus* virus 1 (PaV1). Line length between alleles is directly proportional to the number of base-pair differences between alleles. Each segment along the lines represents a difference of 1 nucleotide. Specific base changes (location based on an alignment of the entire data set and base difference) are indicated between alleles

DISCUSSION

This study is the first to discover PaV1 in the planktonic postlarval stage of *Panulirus argus*, suggesting that larvae may play a role in the dispersal of the virus to new locations. Although we found no evidence of additional post-settlement infection of EBJs deployed as sentinels on the seafloor, the variation in viral sequences within and among lobsters suggests that they can be infected with multiple viral strains. Florida probably receives larvae from *P. argus* populations throughout the Caribbean, and we hypothesize that strain variation in PaV1 infecting postlarvae and EBJs may thus have a biogeographic basis. The results of our laboratory experiment testing for strain mutation in naive, serially exposed lobsters is consistent with the notion that there may be endemic strains of PaV1.

We also discovered a high DNA sequence variability in PaV1. When the PCR products from PaV1-positive postlarvae, EBJs, and juveniles were sequenced, a total of 33 unique alleles were observed. The DNA sequence variation is due to nucleotide differences at 3 microsatellite loci and 10 SNPs. The genetic diversity of PaV1 was surprisingly high for the viral genome region examined; however, sequence variation such as this, in the form of microsatellites or single point mutations is not novel. Relevant to crustaceans, extensive research exists on the variability of white spot syndrome virus (WSSV) in shrimp. Comparison of WSSV genomes from Thailand, Taiwan, and China has revealed that genome size varies due to the presence of several tandem repeats within the DNA sequence (van Hulten et al. 2001, Yang et al. 2001, Chen et al. 2002). The ORF 94 of WSSV from Thailand has 12 different sequence repeat groups (Wongteerasupaya et al. 2003). Variable number tandem repeat regions (VNTRs) (13 microsatellite and 3 mini-satellites) from a different region also have been reported for WSSV

(Shekar et al. 2005), and variations in WSSV ORFs have proven useful as markers in epidemiological studies (e.g. Pradeep et al. 2008). Although we have only documented variability in a single locus of the PaV1 genome, the large number of alleles present at that locus indicates that the variability may be useful for epidemiological studies.

Genetic variation in viruses may also correspond to virulence differences in susceptible hosts. Pathogenicity differences between WSSV isolates are correlated with different genome sizes (Marks et al. 2005, Pradeep et al. 2009); viral isolates with the smallest genome size are more virulent and have increased fitness compared to isolates with larger genomes. Pradeep et al. (2009) reported that isolates with fewer VNTRs in specific ORFs had greater fitness, as determined by relative proportion among isolates when passaged through susceptible hosts. The role of microsatellites in the pathogenicity of PaV1 remains to be determined; however, given the high degree of variability, it seems possible that they could serve as markers for virulence or fitness attributes for the virus. The large amount of variability observed in the microsatellite lengths between isolates makes sense if this portion of the viral genome does not code for a gene, therefore allowing this region to accumulate mutations. Indeed, translation of the viral DNA into amino acids indicated that a portion of the ~500 bp fragment codes for 105 amino acids, with only 3 SNPs that cause amino-acid changes. The region that included the microsatellites does not appear to be within a coding region. Targeting this highly variable region may prove useful in future studies on the population genetics of this virus.

Analysis of samples from the mutation study confirms that the nucleotide variation in viral DNA within individual lobsters is not reflective of a rapidly mutating virus or portion of the virus, but instead reflects co-infection by a few stable viral strains. The duration of the transmission experiment was over 6 mo and most clones from the 5 individuals that acquired the virus via transmission from infected lobsters shared the same major allele found in the lobster whose PaV1-infected hemolymph was used as the donor for the experiment. This is in stark contrast to the allele diversity found in samples collected in the wild. For example, 8 different alleles were observed in just 4 EBJs deployed as sentinels at the Mayor's site.

We were unable to detect local PaV1 infection of EBJ lobsters beyond background levels of PaV1 prevalence in recruiting postlarvae. The EBJs we ob-

tained as postlarvae from the plankton and deployed as sentinels for 2 wk at 7 nursery sites had similar levels of infection as our laboratory-held controls. We attribute this to the episodic, but sometimes high, prevalence of PaV1 infection in postlarvae arriving from offshore, which we used as source animals for both sentinels and controls in our experiment. Yet, these results do not discount the potential for endemic post-settlement infection or sequential infection of lobsters, both of which appear likely based on laboratory infection studies.

It is unclear how the non-feeding postlarvae acquire the virus. The postlarvae were collected from Witham-type collectors on the ocean-side of the Florida Keys and therefore had no contact with juveniles that harbor PaV1 and which reside primarily in the shallow-water nursery on the gulf-side of the Florida Keys. Either the postlarvae were infected by PaV1 while in the plankton, or the virus was transmitted vertically to them from PaV1-infected parents.

Larval *Panulirus argus* have an extremely long planktonic phase of 5 to 7 mo (Goldstein et. al 2008) from the time they are spawned until they metamorphose tens of kilometers offshore into postlarvae that recruit inshore to shallow nursery grounds. Phyllosoma larvae are predators of smaller zooplankton and the PaV1 virus can be transmitted via ingestion in juvenile lobsters. So, it is plausible that PaV1 has an unknown secondary host in the plankton, although attempts to infect 3 other decapods (*Menippe mercenaria*, *Mithrax spinosissimus*, and the congeneric *P. guttatus*) with PaV1 through inoculation have been unsuccessful (Butler et al. 2008). We have demonstrated that waterborne transmission of PaV1 to EBJ lobsters is possible (Butler et al. 2008), so waterborne viruses could potentially infect larvae. PaV1 virions free-floating in the plankton are likely to be in dilute, perhaps non-infectious concentrations unless somehow concentrated as, for example, in marine aggregates (Lyons et al. 2005). However, PaV1 is an unenveloped virus that appears to only be viable in seawater for a few days (C. Li et al. unpubl. data), making waterborne transmission of PaV1 to larvae unlikely.

There are several examples of vertical transmission of viruses in crustaceans. Gill-associated virus (GAV) is transmitted from parent to progeny in the fertilized eggs and nauplii of the black tiger prawn *Penaeus monodon*, spawned from females and males positive for GAV (Cowley et al. 2002). *Macrobrachium rosenbergii* nodavirus (MrNV; the causative agent of white tail disease) and extra small virus (XSV) can be vertically transmitted (Sudhaka-

ran et al. 2007). Rotifers naturally exposed to sediments with WSSV passed the virus to their eggs (Yan et al. 2004), and the virus appears to use vertical transmission in brine shrimp as well (Li et al. 2003). Infectious hypodermal and hematopoietic necrosis virus (IHHNV) of shrimp uses vertical transmission in hosts that survive the acute stages of infection (Motte et al. 2003). We have confirmed the presence of PaV1 in egg-bearing female *Panulirus argus* (authors' unpubl. data) and are currently testing whether the virus can be vertically transmitted to eggs and larvae.

Whether larvae infected with PaV1 can move over the same distances as uninfected larvae remains to be determined, but finding infected postlarvae and infected egg-bearing adults opens the door to this possibility. Adult lobster populations throughout the Caribbean are isolated by distance and bathymetry, but these metapopulations are linked by the dispersal of planktonic larvae. Empirical evidence for long-distance dispersal of *Panulirus argus* larvae includes evidence that Brazilian *P. argus westonii* postlarvae occur occasionally in the waters off Florida (Sarver et al. 2000) and regular collections of *P. argus* in the Cape Verde Islands in the Eastern Atlantic, far from their Western Atlantic sources (Freitas & Castro 2005). Moreover, analysis of mtDNA variation shows that there is no genetic structure of *P. argus* in the tropical and subtropical northwest Atlantic Ocean, consistent with high levels of gene flow among populations (Silberman et al. 1994).

The viral alleles of PaV1 may have utility as markers to demonstrate the connectivity of lobster populations in the Caribbean. Parentage analysis using microsatellite markers, for example, has been performed in a variety of marine species, including oysters (Xiao et al. 2011), clownfish (Planes et al. 2009), sunfish (MacKiewicz et al. 2002), and dolphins (Krützen et al. 2004). Recent investigations of genetic diversity and connectivity between Florida and Caribbean staghorn coral *Acropora cervicornis* found shared mtDNA haplotypes between the western Caribbean and Florida despite the fact that the planktonic larval duration of *A. cervicornis* is only about 4 d (Hemond & Vollmer 2010). Potential sources of PaV1 to the Florida Keys include areas considered 'upstream' by current larval dispersal models (Butler et al. 2011). We are currently examining the biogeographic distribution of PaV1 alleles in the Caribbean to determine if PaV1 alleles can be used as biological 'tags' to test and validate simulation models of lobster dispersal, and hence population and disease connectivity in the Caribbean Sea.

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