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Low genetic diversity of the putatively introduced, brackish water hydrozoan, *Blackfordia virginica* (Leptothecata: Blackfordiidae), throughout the United States, with a new record for Lake Pontchartrain, Louisiana

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Abstract.—Despite first being described from Virginia, the widely distributed brackish water hydrozoan *Blackfordia virginica* is often hypothesized to have been introduced from the Black Sea to the United States. However, the alternative view that *B. virginica* was introduced to the Black Sea also persists in the literature. This study investigates the population structure of *B. virginica* in the United States to assess the directionality and/or the number of introduction events. During 2009 and 2010, estuaries were sampled from Delaware to Louisiana for brackish water hydromedusae. Nineteen samples of *Blackfordia virginica* were collected from four localities, including a channel running between St. Catherines Island and Lake Pontchartrain, Louisiana, a region for which it had not been reported prior to this study. We PCR amplified and sequenced two mitochondrial markers (COI & 16S), and one nuclear marker (ITS1). We compared data from individuals collected on the east coast of the United States with individuals collected in California. This revealed low diversity (two haplotypes with a maximal p-difference of 0.03% for COI and just a single haplotype for 16S) and no unique haplotypes at any locality. Low genetic variability, shared haplotypes in disparate localities, and a lack of unique haplotypes in any population are consistent with a founder effect, suggesting a single introduction and subsequent spread throughout the United States.

Keywords: founder effect, genetic variation, Hydrozoa, invasive species, medusa

Species in the cnidarian class Hydrozoa are relatively inconspicuous and may, therefore, be overlooked in invasion biology. However, the ecological impact of these organisms could be great because they reproduce asexually, often survive in a wide range of salinities, and are predators of fish larvae, crustaceans, and other planktonic and benthic organisms (Bouillon et al. 2006). As a result, neglecting Hydrozoa could cause inaccuracies in ecological assessments of introduced species (Bouillon et al. 2004).

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Blackfordia virginica Mayer, 1910 was first described from specimens taken in an estuarine environment in the United States (U.S.) near Hampton Roads, Virginia (Mayer 1910). Within the U.S., it has subsequently been found in the Napa and Petaluma Rivers in San Francisco Bay, California (Mills & Sommer 1995), Coos Bay in Oregon (Mills & Rees 2000), as well as Delaware Bay (Cronin et al. 1962) and is presumed to have been introduced to the United States from the Black Sea region (Mills & Sommer 1995). The hypothesis that *B. virginica* is native to the Black Sea and was introduced to Virginia through shipping traffic prior to its discovery there appears to stem from an observation of high abundance of *B. virginica* in the Mandra swamp on the Bulgarian coast (Mills & Sommer 1995). An alternative hypothesis is that *B. virginica* is native to Virginia, where it was first collected and subsequently introduced to the Black Sea (Leppäkoski et al. 2009). Internationally, *B. virginica* has also been reported in the Cananeia, Guaratuba, and Babitonga Bays, Brazil (Bardi & Marques 2009), Argentina and Uruguay (Genzano et al. 2006), Mexico (Álvarez Silva et al. 2003), Portugal and Spain (Chicaro et al. 2009), the Caspian Sea, which boarders Azerbaijan, Iran, Kazakhstan, Russia and Turkmenistan, (Leppäkoski et al. 2009), the Loire estuary in France, the Mira estuary in Portugal, Nova Scotia, Canada (Moore 1987), Lake Belona in Romania, the South China Sea and East China Sea in the Fujian Province (Zhang 1982, Lin & Zhang 1990), and the Ganges and Vasishta Godvari estuaries in India (Sastry & Chandramohan 1989). No genetic studies have thus far been carried out to address the dynamics of any population of *B. virginica*.

This study investigates the population structure of *B. virginica* in the U.S. to shed light on the conflicting hypotheses on its provenance. It also provides the first report of *B. virginica* near Lake Pontchartrain, Louisiana. We collected and sequenced 19 individuals from four locations around the United States: Delaware, Virginia, Louisiana, and California. We sequenced two mitochondrial (16S & COI) and one nuclear rDNA regions (ITS1). Because samples of *B. virginica* from the Black Sea were not available for sequencing for this study, a direct comparison of the two populations was not possible. Instead, genetic variation of *B. virginica* in the U.S. was compared with that of other hydrozoan species. While not a perfect test, a comparatively lower level of genetic variation relative to other hydrozoan species suggests that populations of *B. virginica* in the U.S. was founded on a single introduction event and has subsequently spread. Furthermore, the genetic structure of *B. virginica* in the U.S. documented here is an important contribution as it provides baseline data for future studies of populations in other geographic regions and insight into invasion patterns of a widely distributed species.

Materials and Methods

Collection methods.—Specimens of *Blackfordia virginica* were collected with plankton tows in brackish estuaries. Three disparate locations in the U.S. were sampled: the Mattaponi River, Virginia; Slaughter Beach, Delaware, and Lake Pontchartrain, Louisiana (Table 1). *Blackfordia virginica* from San Francisco Bay was provided to us by Dr. Mariah Meek from the University of California, Davis. Our collections include all geographic regions in which *B. virginica* had previously been reported in the literature, with the exception of Hampton Roads, Virginia and Coos Bay, Oregon. A new collection location in Louisiana was included. The newly reported *B. virginica* from Louisiana was preserved in a formalin and seawater solution. We examined morphological

Table 1.—Collection information for *Blackfordia virginica* from 2009–2010.

Date	Location	*n*	Salinity (ppt)	Latitude	Longitude
11 Jun 10	Mattaponi River, Virginia	3	5–23	36°32′26.66″N	76°47′21.67″W
9 Jun 09	Lake Pontchartrain, Louisiana	10	5–10	30°05′34.43″N	89°46′37.63″W
18 Jun 10	Slaughter Beach, Delaware	3	18–20	38°56′06.26″N	75°19′26.24″W
8 Aug 09	San Francisco Bay, California	4	Unknown	–	–

characteristics to determine whether there was phenotypic variation in the *B. virginica* collected at this site compared to previous descriptions of the species. These characters include quantifying the number of tentacles, the absence or presence of pigmentation between tentacles, the number of statocysts between tentacles, the mouth, oral arms, and radial canals were quantified and photographed. Photographs of nematocysts were taken using SPOT v. 4.6 software (Diagnostic Instruments). Measurements were taken using a Nikon E80i compound microscope viewed with a 100X objective.

DNA extractions were performed using a Qiagen DNeasy Blood and Tissue Kit® (Qiagen, Valencia, California). A total of 19 individuals were sequenced at three relatively quickly evolving loci: mitochondrial 16S and COI, as well as the nuclear ribosomal marker ITS1, using previously published primers (Table 2). We performed Polymerase chain reaction (PCR) amplification in a 20 μl reaction. Mixes were the same for each gene and were composed as follows: 1x reaction buffer, 1x BSA, 0.08 mM of dNTPs, 0.032 mM of MgCl, 0.24 μM of each primer, 1 unit of taq and 2 μl of genomic DNA. The same cycling protocol was used for 16S and COI: 95°C for 7 minutes followed by 40 cycles of 95°C for 30 seconds, 48°C for 30 seconds, 72°C for 1 minute, followed by an extension of 72°C for 5 minutes, and a 10°C holding temperature. The cycling protocol for ITS1 was the same with the exception of a 60°C annealing temperature. We verified PCR amplification on a 1.5% electrophoresis gel stained with ethidium bromide.

We directly sequenced product in both directions via Sanger sequencing, using BigDye® terminator v. 3.1. Sequences were aligned forward and reverse by the sample name handle and disputed nucleotide calls were corrected by comparing peaks on the chromatograms using Sequencher software (v. 4.9, Gene Codes Co., Ann Arbor, Michigan). Text files of the consensus sequences were exported and uploaded into Seaview v. 4.0 (Guoy et al. 2010) for

Table 2.—List of primers used to amplify mitochondrial and nuclear regions.

Primer Name	Loci	Sequence	Citation
jlITS1F	ITS1	5′GGTTTCCGTAGGTGAACCTGCGGAAGGATC′3	Dawson & Jacobs 2001
jlITS1R	ITS1	5′CGCACGAGCCGAGTGATCCACCTTAGAAG′3	Dawson & Jacobs 2001
16s.Cunningham.F.1mod	16S	5′ACGGAATGAACTCAAATCATGTAAG′3	Bridge et al. 1995
16s.Cunningham.R.2	16S	5′TCGACTGTTTACCAAAAACATA′3	Bridge et al. 1995
dgLCOI490	COI	5′GGTCAACAAATCATAAAGAYATYGG′3	Folmer et al. 1994
dgHCO2198	COI	5′TAAACTTCAGGGTGACCAAARAAYCA′3	Folmer et al. 1994

alignment and nucleotide comparison. Nucleotide point mutations were re-examined in Sequencher to confirm that base calls were accurate. ITS1 must be specially considered because it is present in multiple copies and can exhibit multiple alleles visualized as a double peak at a base; therefore, sequences were duplicated before making calculations. One common sequence was apparent in all individuals and counted as the first allele. In three individuals, dual peaks were seen at two positions, and these were used in the calculations as the second allele for those individuals. For the homozygous individuals, the common allele was counted twice. For the heterozygous individuals, the common allele was counted once and the alternative allele was counted once.

No samples were available from the Black Sea for direct comparison. Therefore, the genetic variation (percent of base differentials) of *B. virginica* collected in the U.S. was compared to that of other hydrozoan species with sequence data available in GenBank (www.ncbi.nlm.nih.gov/genbank/GenbankSearch.html). To accomplish this, a file of 16S and COI sequences was created for all hydrozoan species present in GenBank with five or more sequences available. ITS1 was excluded due to a lack of available sequences. Sequences were aligned using MAFFT (v. 6, Copyright © 2011 Kazutaka Katoh) and analyzed in PAUP* (v. 4.0a133, Swofford 2002) to calculate pairwise percent variation (p) and average intraspecific variation for each hydrozoan species.

Results and Discussion

Morphological description.—The specimens collected in Lake Pontchartrain, Louisiana had four radial canals and a mouth with four perradial oral arms (Fig. 1A, C). The mesoglea was thick at the apex and thinner at the umbrellar margins (Fig. 1B). The specimen had a total of 84 tentacles and one to two statocysts between each tentacle (Fig. 1D). One set of tentacles had three statocysts between them. Pigment granules were present on the intertentacular marginal region (Fig. 1E). Small, oval shaped nematocysts were observed on the tentacles, which were identified as microbasic mastigophores by using the description from Mariscal (1974) (Fig. 1F, G). A total of 10 nematocysts were measured. Nematocyst length ranged from 8.0 μm to 12.7 μm with an average of 10.4 μm. Width of the nematocysts ranged from 3.1 μm to 5.1 μm with an average of 3.6 μm. This individual was similar to previous descriptions of *B. virginica* from Mayer (1910) and Mills & Sommer (1995) and did not exhibit any obvious phenotypic variation.

Molecular description of Blackfordia virginica *in the United States.*—Overall we found little genetic variation at any of the three loci within the 19 analyzed individuals. At the 16S locus, the average percent variation was 0%. For COI the average percent variation for *B. virginica* collected in the United States was 0.03% with a maximum percent variation of 0.16% and a minimum percent variation of 0%. Two haplotypes were the result of a single point mutation occurring at the same base. This haplotype was found in one individual from Louisiana and one individual from Virginia (Fig. 2). The remaining 17 individuals contained 0% average genetic variation. Ribosomal ITS1 had the highest variation with an average percent variation at 0.42% with a maximum of 4.73% and a minimum of 0%. Two haplotypes were present resulting from two point mutations with two bases between them. Individuals were either homozygous for the common haplotype or heterozygous for the less common haplotype. Of the three heterozygous individuals, two were collected in Louisiana and one was collected in Virginia (Fig. 3).

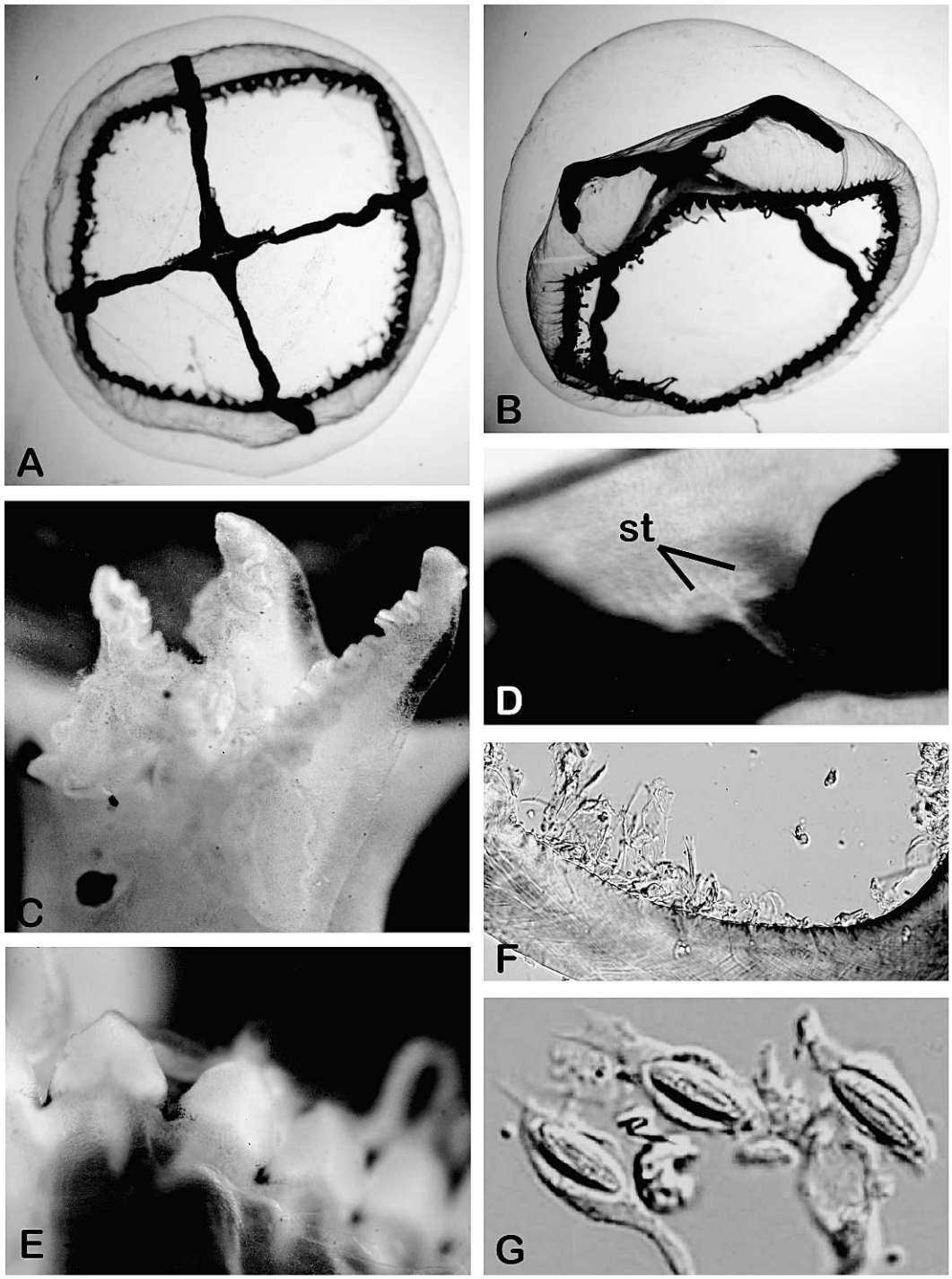

Fig. 1. *Blackfordia virginica*. A, specimen from Lake Pontchartrain, Louisiana; B, specimen showing thicker mesoglea in apex than around umbrellular edge; C, specimen showing square mouth surrounded by four oral arms; D, two statocysts (st) between tentacles; E, granules of dark pigmentation between tentacles; F, tentacle showing discharged nematocysts; G, microbasic mastigophore nematocysts. Size information provided in text.

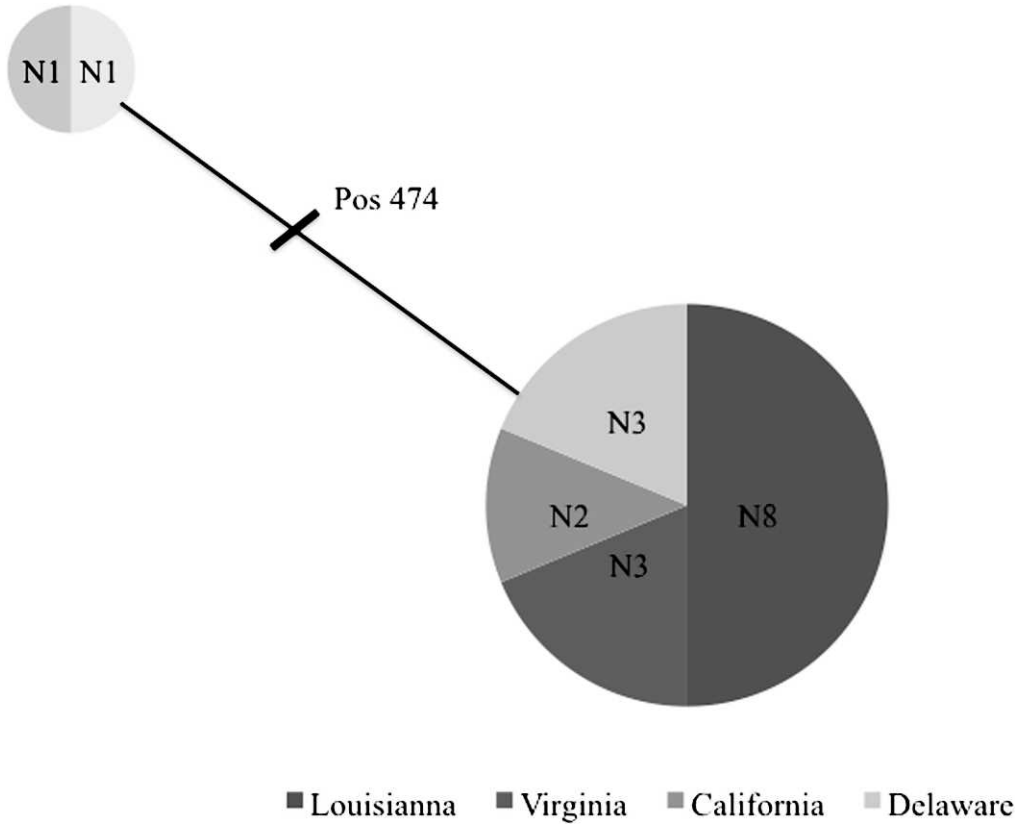

Fig. 2. Cluster diagram showing haplotypes and locations of *Blackfordia virginica* for COI. N = number of individuals; Pos = position in which bases differ.

Intraspecific variation in mitochondrial 16S and COI.—The *Blackfordia virginica* population in the U.S. exhibited low genetic variation and few haplotypes, suggesting a founder effect. If *B. virginica* were native to the United States, we would expect to see higher genetic variation here and thus conclude that *B. virginica* has likely been introduced to the U.S. To firmly test this conjecture, *B. virginica* populations found in the U.S. should be compared to those collected in the Black Sea as well as other continents. Assuming that the U.S. populations are indeed introduced, there is evidence that *B. virginica* collected in Lake Pontchartrain, Louisiana is the result of pan-mixing between brackish estuaries rather than a re-introduction from the Black Sea or another international population. If multiple introduction events had occurred, we would expect to see unique haplotypes in different geographical locations. No unique haplotypes are found in the Lake Pontchartrain or any other population.

Comparison of Blackfordia virginica *to other hydrozoan species.*—To assess whether the genetic variation is indicative of an introduction event, or is the norm for hydrozoans, 16S and COI sequences available in the GenBank nucleotide database were utilized for comparison. For both genetic markers, *B. virginica* remained at the lowest range of genetic variation compared to mitochondrial 16S sequences from 23 species of Anthoathecata (390 sequences) and seven species of Leptothecata (99 se-

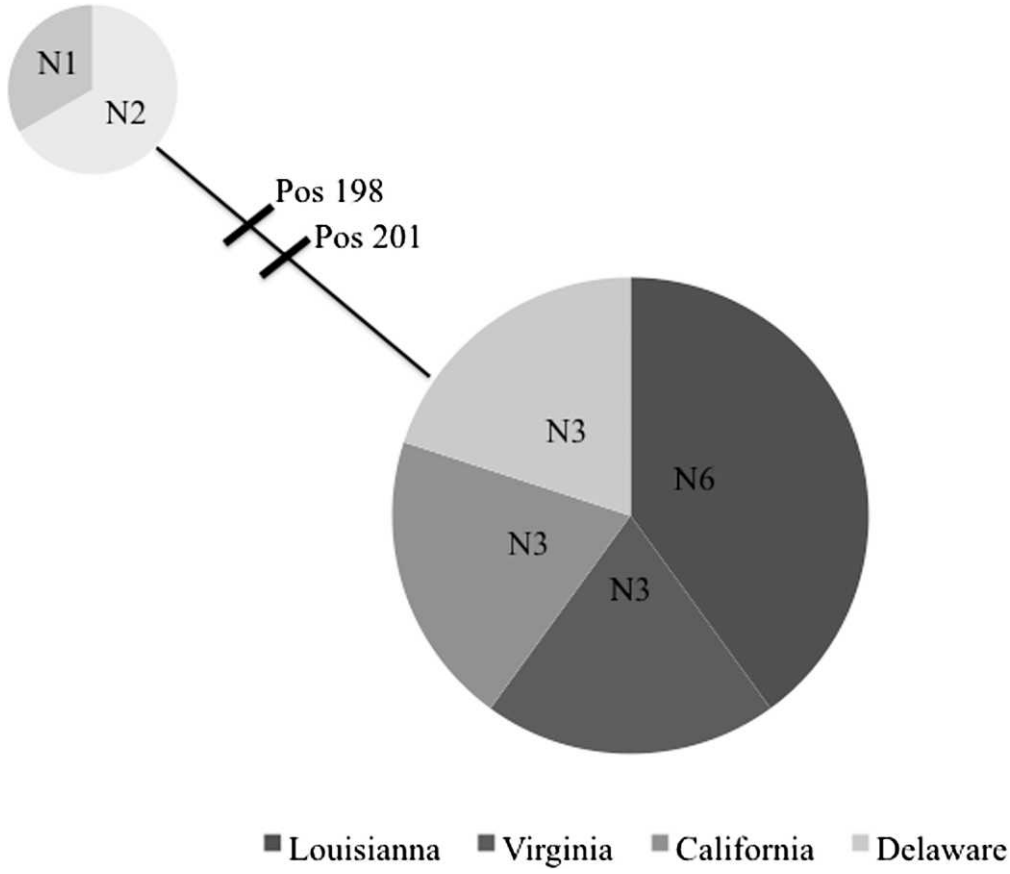

Fig. 3. Cluster diagram showing the haplotypes and locations of *Blackfordia virginica* for ITS1. Abbreviations as in Fig. 2.

quences). The average intraspecific variation for this marker ranged from 7.8% found in *Clytia gracilis* to 0% in *B. virginica* (Fig. 4). There was no apparent correlation between overall variation and order, habitat (marine, brackish, or freshwater), or life cycle (Tables 3, 4). For species in which both COI and 16S sequences were available, COI exhibited a higher average genetic variation comparatively, which is also seen in our analysis of *B. virginica*.

We compiled COI sequences for four species of Siphonophora (23 sequences), one species of Limnomedusae (6 sequences), four species of Leptothecata (80 sequences), and six species of Anthoathecata (159 sequences). The mean intraspecific variation ranged from 10.9% in *Hydra viridissima* to 0% in *Craspedacusta sowerbii* (Fig. 5), which is a freshwater jellyfish (Limnomedusae) also presumed to be introduced to the U.S. (Dumont 1994, Jankowski et al. 2008). *Blackfordia virginica* had the second lowest level of genetic variability for COI (0.03%). Both *Hydra viridissima* and *Clytia gracilis* appear to be beyond the expected range for a species. Because it is difficult to identify morphological differences within the genus *Clytia* due to a lack of knowledge about their life cycle and the way their environment shapes prominent taxonomic characteristics (Calder 1991, Lindner et al. 2011), the

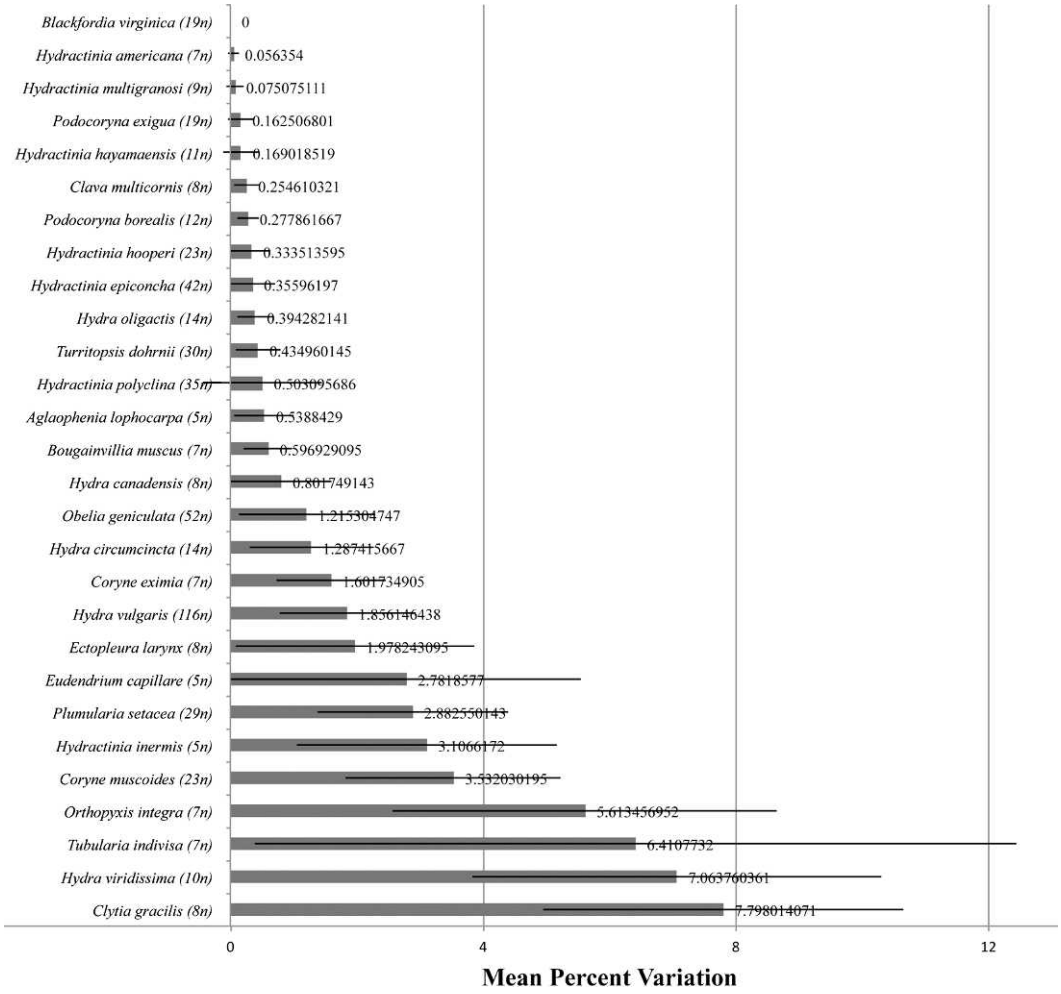

Fig. 4. Average intraspecies percent variation at 16S locus of Hydrozoa. Error bars represent standard deviation (n = number of individuals).

high genetic variation could be a result of cryptic species being included within the sequence data. High genetic variation has been previously reported in *Hydra viridissima* (Kawaida et al. 2010, Martinez et al. 2010), but both of these studies suggest that multiple species exist in what they also refer to as the "*viridissima* group."

The low genetic variation is consistent with an introduction event, especially when coupled with the haplotype data. In Leptothecata, to which *B. virginica* belongs, the average variation in 16S and COI are 3.29% and 3.39%, respectively. We found 0% variation for 16S, and the maximum variation for COI was 0.16%. It is worth emphasizing that identical haplotypes are found, even though *B. virginica* was collected from disparate locations (east and west coast of the U.S.). Thus, this finding is inconsistent with what would be expected in the case of multiple introductions. The lack of unique haplotypes also suggests that sub-populations within the U.S. have not been isolated for long periods of time. Nevertheless, in the

Table 3.—16S data of mean percent intraspecific genetic variation for hydrozoan species. Key for life history: C = colonial hydroid, M = medusa present, Med = medusoid present, S = solitary hydroid. Key for salinity: Brac = brackish, F = fresh, M = marine.

Species	*n*	Order	Mean % variation	*SD*	Life history	Salinity
Clytia gracilis	8	Leptothecata	7.80	2.85	C/M	M
Hydra viridissima	10	Anthoathecata	7.06	3.24	S	F
Tubularia indivisa	7	Anthoathecata	6.41	6.03	S/Med	M
Orthopyxis integra	7	Leptothecata	5.61	3.03	C/Med	M
Coryne muscoides	23	Anthoathecata	3.53	1.70	C	M
Hydractinia inermis	5	Anthoathecata	3.11	2.06	C/Med	M
Plumularia setacea	29	Leptothecata	2.88	1.52	C	M
Eudendrium capillare	5	Anthoathecata	2.78	2.77	C	M
Ectopleura larynx	8	Anthoathecata	1.98	1.89	C/Med	M
Hydra vulgaris	116	Anthoathecata	1.86	1.08	S	F
Hydra circumcincta	14	Anthoathecata	1.29	0.98	S	F
Obelia geniculata	52	Leptothecata	1.22	1.08	C	M
Hydra canadensis	8	Anthoathecata	0.80	0.81	S	F
Bougainvillia muscus	7	Anthoathecata	0.60	0.38	C/M	M
Aglaophenia lophocarpa	5	Leptothecata	0.54	0.46	C	M
Hydractinia polyclina	35	Anthoathecata	0.50	0.95	C	M
Turritopsis dohrnii	30	Anthoathecata	0.43	0.36	C/M	M
Hydra oligactis	14	Anthoathecata	0.39	0.28	S	F
Hydractinia epiconcha	42	Anthoathecata	0.36	0.35	C	M
Hydractinia hooperi	23	Anthoathecata	0.33	0.31	C	M
Podocoryna borealis	12	Anthoathecata	0.28	0.17	C/M	M
Clava multicornis	8	Anthoathecata	0.25	0.20	C	M/Brac
Hydractinia hayamaensis	11	Anthoathecata	0.17	0.29	C	M
Podocoryna exigua	19	Anthoathecata	0.16	0.20	C/M	M
Hydractinia multigranosi	9	Anthoathecata	0.08	0.14	C	M
Hydractinia americana	7	Anthoathecata	0.06	0.09	C	M
Coryne eximia	7	Anthoathecata	0.02	0.86	C/M	M
Blackfordia virginica	19	Leptothecata	0		C/M	Brac

Table 4.—COI data of mean percent intraspecific genetic variation for hydrozoan species. Key for life history: C = colonial hydroid, M = medusa present, Med = medusoid, S = solitary hydroid. Key for salinity: Brac = brackish, F = fresh, M = marine.

Species	*n*	Order	Mean % variation	*SD*	Life history	Salinity
Hydra viridissima	17	Anthoathecata	11.75	6.32	S	F
Clytia gracilis	7	Leptothecata	10.82	4.77	C/M	M
Hydra vulgaris	69	Anthoathecata	5.87	3.65	S	F
Hydra circumcincta	14	Anthoathecata	4.04	2.80	S	F
Obelia geniculata	51	Leptothecata	2.31	2.17	C	M
Hippopodius hippopus	6	Siphonophorae	3.08	1.55	C	M
Hydra utahensis	5	Anthoathecata	1.48	0.98	S	F
Agalma elegans	5	Siphonophorae	1.37	0.41	S	M
Agalma okeni	5	Siphonophorae	0.61	0.22	S	M
Hydra robusta	7	Anthoathecata	0.65	0.63	S	F
Nemopsis bachei	62	Anthoathecata	0.25	0.36	C/M	Brac/M
Obelia longissima	5	Leptothecata	0.41	0.16	C	M
Nanomia cara	7	Siphonophorae	0.23	0.08	C	M
Blackfordia virginica	19	Leptothecata	0.03	0.05	C/M	Brac/M
Craspedacusta sowerbii	28	Limnomedusae	0	0	S/C/M	F

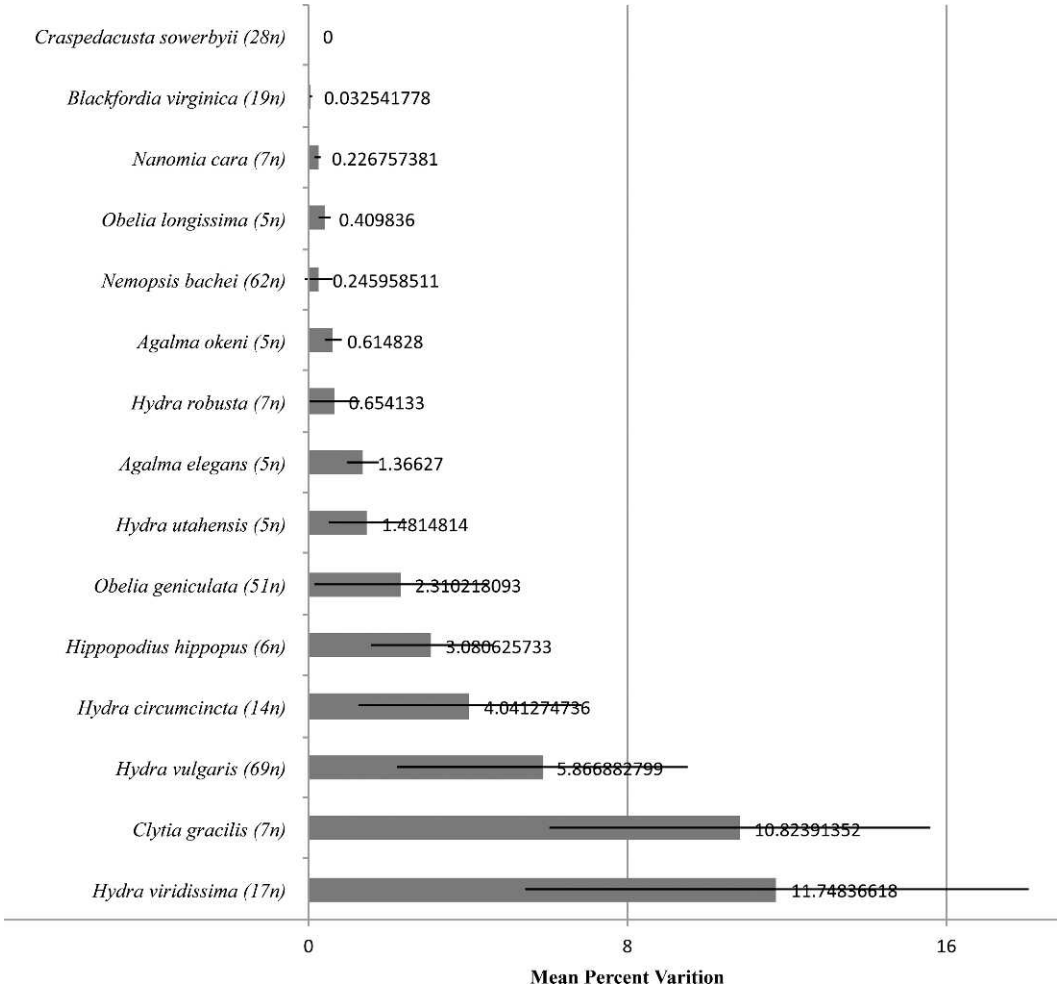

Fig. 5. Average intraspecies percent variation at the COI locus of Hydrozoa. Error bars represent standard deviation (n = number of individuals).

absence of a more complete sampling, these results must be taken as somewhat tentative. To truly understand the population history of this interesting hydrozoan species, individuals should be collected from multiple continents on which they are found.

Acknowledgments

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