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Molecular phylogenetics of the *Neanthes acuminata* (Annelida: Nereididae) species complex

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Abstract

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The *Neanthes acuminata* (Nereididae) species complex is a broadly distributed group of marine benthic polychaetous annelids that is known by many names around the world and comprises at least four species. They are the only nereidids known that show exclusively male parental care. The female dies after laying her eggs in a common mucoid tube where they are fertilized, and the male incubates the eggs until the young leave the tube. All of the species in the *N. acuminata* complex are identical in their morphological characteristics and they all possess a similar number of segments and paragnath distribution and similarly shaped parapodia. However, populations from the U.S. East Coast, southern California, Hawaii and Portugal differ in chromosome number. Eye and egg colour also vary among populations—some worms in southern California have red eyes and produce bright yellow/orange eggs, while others have black eyes and produce pale yellow eggs. These variations suggest that *N. acuminata* may represent multiple evolutionarily significant units. Clarification of the phylogenetic relationships among lineages in this species complex will provide a framework for studying character evolution and revising taxonomy within this intriguing group of nereidids. To that end, we sequenced regions of one nuclear and two mitochondrial genes from worms sampled from multiple sites in North America (southern California, Mexico and Connecticut), the central Pacific (Hawaii) and Europe (Germany, Portugal and the UK). Maximum likelihood and Bayesian analyses of these data clarify relationships in this complex and show that worms sampled from California and Mexico represent two geographically intermingled subclades. These two subclades are congruent with eye and egg colour data; one subclade consists of red-eyed worms, the other consists of black-eyed worms. Furthermore, we found evidence that individuals representing these subclades can occasionally be found at the same locality.

Keywords

Neanthes caudata, *Neanthes arenaceodentata*, polychaete, phylogenetic relationships, morphs, COI, 16S, ITS1.

Introduction

The polychaete *Neanthes acuminata* (Ehlers, 1868) (Annelida: Nereididae) species complex is cosmopolitan in distribution and comprises at least four species (Weinberg et al., 1990). *Neanthes acuminata* is the only valid scientific name for this group. It is known by this name from New England to North Carolina (Day, 1973). The southern California population was initially referred to the European species *N. caudata* (delle Chiaje, 1841) (Reish, 1957), which was later considered a synonym of *N. arenaceodentata* Moore, 1903 by Pettibone (1963). This name was applied to the California population by Reish and Alosi (1968), but Day (1973) considered both *N. caudata* and *N. arenaceodentata* synonyms of *N. acuminata*. *Neanthes cricognatha* (Ehlers, 1904), also considered part of the complex, is known from India and Hong Kong (Fauvel, 1950) and Australia and New Zealand. References to the literature concerning the populations used in this study are presented in Table 1. For convenience in this paper, *N. acuminata* refers to samples from New England; *N. caudata* to samples from Portugal, and *N. arenaceodentata* to samples from southern California, Mexico and Hawaii. All members of this species complex, except *N. cricognatha*, have been cultured through several generations in the laboratory at California State University, Long Beach (CSULB) by coauthor Reish (DJR). All are morphologically identical, with small conical paragnaths covering both rings of the proboscis and neuropodial heterogomph compound chaetae with a long blade terminating with a hook. Reproduction is unique in that the female reproduces once, but the male, which takes care of the embryos through the 21st segmented stage, is capable of reproducing as many as nine times (Reish et al., 2009). The

Table 1. Selected references to literature concerning the populations used in this study

Neanthes arenaceodentata:
Los Angeles Harbour Reish, 1956 [as *N. caudata*], Crippen and Reish, 1967, Reish, 1972
Venice, California: Winchell, et al. 2010.
Alamitos Bay: Reish, 1964 [as *N. caudata*], 1972
San Gabriel River, Reish, 1972, Oshida, et al. 1976.
Newport Bay: Reish, 1972
Punta Banda, Mexico: Díaz-Castañeda and Rodréguez-Villanueva, 1998
Hawaii: Bailey-Brock, et al., 2002.
Neanthes acuminata
Connecticut: Day, 1973, Weinberg, et. al., 1990
Neanthes caudata
Portugal: Fauvel, 1923, Bellan, 1967

members of the species complex differ from each other on the basis of chromosome number: *Neanthes acuminata* (2N = 22), *N. arenaceodentata* (2N = 18), Hawaiian *N. arenaceodentata* (2N = 28) (Weinberg et al., 1990*), N. caudata* (2N = 18) (Reish, unpublished) and behaviour (Sutton et al., 2005). Worms prefer to mate with worms collected from the same population. Males and females from southern California and New England, as expected, were aggressive to each other and failed to mate (Sutton et al., 2005). The chromosome number is unknown for *N. cricognatha*, which is not a part of the present study.

Materials and Methods

Taxon sampling

Neanthes acuminata was collected from the Connecticut intertidal zone by J. D. Hardege in 2004 and transported to CSULB where it was cultured for more than 10 generations before the culture was terminated. Collections from southern California were collected by DJR with exception of those from Venice Lagoon which had previously been cultured by Christopher J. Winchell at the University of California, Los Angeles. Collections from Estero Punta Banda and Bahia de San Quintin, Baja California were preserved in 70% ethanol by Maricarmen Necoechea and shipped to DJR. The Hawaiian specimens were collected by Bruno Pernet and shipped live to CSULB. Culturing these worms was unsuccessful and living specimens were preserved in 70% ethanol prior to death. Living specimens, except as noted above, were shipped by overnight express to the University of Hull and Southern Illinois University. Additional data on location, date of collection, culture history and locality are given in Table 2. Specimens from laboratory populations have been deposited in the Los Angeles County Museum of Natural History under the following catalog numbers: LACM-AHF 6194 (Reish lab), 6195 (L.A. Harbour), 6196 (Venice Canals), 6197 (Alamitos Bay), 6198 (San Gabriel River), 6199 (Newport Bay), 6200 (Hawaii) and 6201 (Faro, Portugal).

Culture methods were the same for all populations. Cultures were established by pairing a female, as determined by the presence of large eggs in her coelom, with a sexually unknown worm. A behavioral response was used to determine a male. Same sexes fight and opposite sexes lie alongside one another (Reish and Alosi, 1968). Males cannot be determined by the presence of sperm as in epitokal nereidids. Pairs were placed in a petri dish containing normal sea water and fed rehydrated dried *Enteromorpha* sp. and constructed a common mucoid tube. The female lays her eggs within the tube where they are fertilized. The female dies after egg laying or is eaten by the male. The male incubates the developing embryos by his body undulations, which refresh the water within the tube. After three to four weeks, the young worms (~21 segments) emerge from the parent's tube and commence feeding (Reish, 1957). There is no pelagic larval stage. Established populations were maintained in aerated 15-gallon (57 liters) aquaria containing 10 gallons (38 liters) of seawater. Approximately 100 juvenile worms were used to establish a population in an aquarium. Worms were fed weekly with commercial rabbit food that was soaked in seawater prior to use, stirred and the supernatant fluid added to the aquarium. Aquaria were drained and cleaned monthly. Worms reproduced within the aquaria and specimens were removed as needed.

DNA Extraction, PCR, and Sequencing

DNA was extracted from tissue samples using a DNeasy kit (Qiagen) according to manufacturer's instructions. Regions of two mitochondrial markers – cytochrome oxidase subunit I (COI or *cox1*) and the 16S ribosomal subunit (rrnL) – and one nuclear marker – internal transcribed spacer 1 (ITS1) – were amplified via PCR using HotStar Master Mix (Qiagen) (half reactions) using primer pairs (COI—Folmer et al., 1994; 16S—Geller et al., 1997; ITS1: ITS III and ITS VIII; Palumbi, 1996). PCR thermal cycling parameters were as follows: 95˚C (15 minutes) for enzyme activation, followed by 35 cycles of 95˚C (45 seconds), 40˚C (45 seconds) and 68˚C (1 minute) and a final terminal extension cycle of 68˚C for 7 minutes. PCR products were purified using a MinElute Gel Purification Kit (Qiagen) and both strands were sequenced on an ABI 3730xl automated sequencer.

Molecular Data Set Construction

COI, 16S and ITS1 sequences were downloaded from the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) for members of three other species; these were used as outgroups for our analyses. Two of these species were (like *Neanthes*) members of Nereididae—*Namalycastis jaya* Magesh, Kvist and Glasby (2012) (GenBank accession numbers: HQ456363 [COI] and HM138706 [16S]) and *Platynereis dumerilii* (Audouin and Milne-Edwards, 1834) (GenBank accession numbers [complete mitochondrial genome]: AF178678) A non-nereidid phyllodocid (*Nephtys sp.* 'San Juan Island' YV-2008, GenBank accession number [complete mitochondrial genome]: EU293739) was used as a distant outgroup to root the tree. These taxa were chosen because both COI and 16S data were available from the same

Table 2. Collection, culture and analysis location data on *Neanthes acuminata* complex

Collection locality	Collection date	Eye/ova color	# lab generations	Lab
Connecticut[1]	2002	Black/pale	~20	Hull
Venice, California	2008	Black/pale	~10	Hull, SIU
Los Angeles Harbour[2]	1964	Red/orange	200+	Hull, SIU
Los Angeles Harbour[1,3]	2008	Red/orange	~12	Hull. SIU
Alamitos Bay	2011	Black/pale	~4	Hull, SIU
San Gabriel River	2008	Black/pale	~12	Hull, SIU
Newport Bay	2004	Red/orange	~20	Hull, SIU
Bahia de San Quintin and Estero	2010	Unknown	Not cultured	Hull, SIU
Punta Banda, Baja California	2010	Unknown	Not cultured	
Oahu, Hawaii	2011	Black/pale	Not cultured	SIU
Portugal	2009	Black/pale	~8	Hull, SIU
Humber Estuary, UK[4]				Hull
Bremerhaven Estuary, Germany[4]				Hull

[1]No longer in culture
[2]Collected from the inner harbour (Reish lab)
[3]Collected from the outer harbour
[4]*Nereis diversicolor*

voucher specimen for all three species (ITS1 data from the outgroups were not included in our analyses; ITS1 sequences from species outside the *Neanthes acuminata* complex could not be aligned with ingroup ITS1 sequences).

Sequence Alignment and Phylogenetic Analyses

Sequence contigs were assembled and edited using Sequencher 5.1 (GeneCodes, Ann Arbor, Michigan), aligned with MUSCLE v3.8.31 (Edgar, 2004) and concatenated in Mesquite (Maddison and Maddison, 2010). ITS1 sequences from the outgroups (*Namalycastis* and *Platynereis*) were highly divergent from *Neanthes acuminata* ITS1 sequences, leading to spurious preliminary alignments. As a result, we excluded outgroup ITS1 data from the data matrices prior to alignment. Preliminary analyses suggested that the individual loci supported topologically concordant phylogenies, so data from the three individual loci were concatenated into two data matrices—a "full" data set (comprising all specimens for which at least one locus—COI, 16S or ITS1—was sequenced), and an "all three genes" data set (comprising all specimens for which COI, 16S and ITS1 sequences were generated). Four data partitioning schemes were evaluated for these data sets: 1) no partitioning (i.e., one data subset), 2) partitioned by gene (three data subsets), 3) partitioned by gene, with first and second codon positions of COI separated from third codon positions (four data subsets: COI positions 1 and 2, COI position 3, 16S and ITS1) and 4) partitioned by gene with COI partitioned by codon (five data subsets; ITS1, 16S, COI 1st, 2nd and 3rd codon positions). Best-fitting substitution models were chosen using jModelTest v2.1.1 (Darriba et al., 2012) and the best-fitting partitioning scheme was chosen using a second-order correction of the Akaike information criterion (AICc) and the Bayesian Information Criterion (BIC). Partitioned maximum likelihood analyses were performed with GARLI 2.0 (Zwickl, 2006). The ML tree search consisted of 10 searches (5 with random starting trees and 5 with stepwise starting trees, each with 100 search replicates); ML bootstrap analysis in GARLI 2.0 comprised 100 pseudoreplicates, each with random starting trees and 10 search replicates. Bayesian analyses were performed with MrBayes v3.2.1 (Ronquist and Huelsenbeck, 2003), with four independent runs of four chains each, temperature set to 0.05 to improve mixing, and the run automatically terminated when a topological convergence diagnostic (the average standard deviation of split frequencies) dropped below 0.01. For Bayesian analyses, data were unpartitioned or partitioned by gene and codon position, as described for ML analyses.

Results

DNA was extracted from a total of 115 specimens. Due to difficulty in PCR amplification of some loci from some specimens and missing sequences for some loci for some outgroup taxa, the full data set comprises a substantial amount of missing data (Table 2). GenBank numbers for the sequences generated in this study are COI: KJ539071 - KJ539141, 16S: KJ538962 - KJ538996, ITS1: KJ538997 - KJ539070. The full and "all three genes" data matrices are available on request to FEA.

The best-fitting models for each of the partitions were as follows—COI 1st positions: 000010+G, COI 2nd positions:

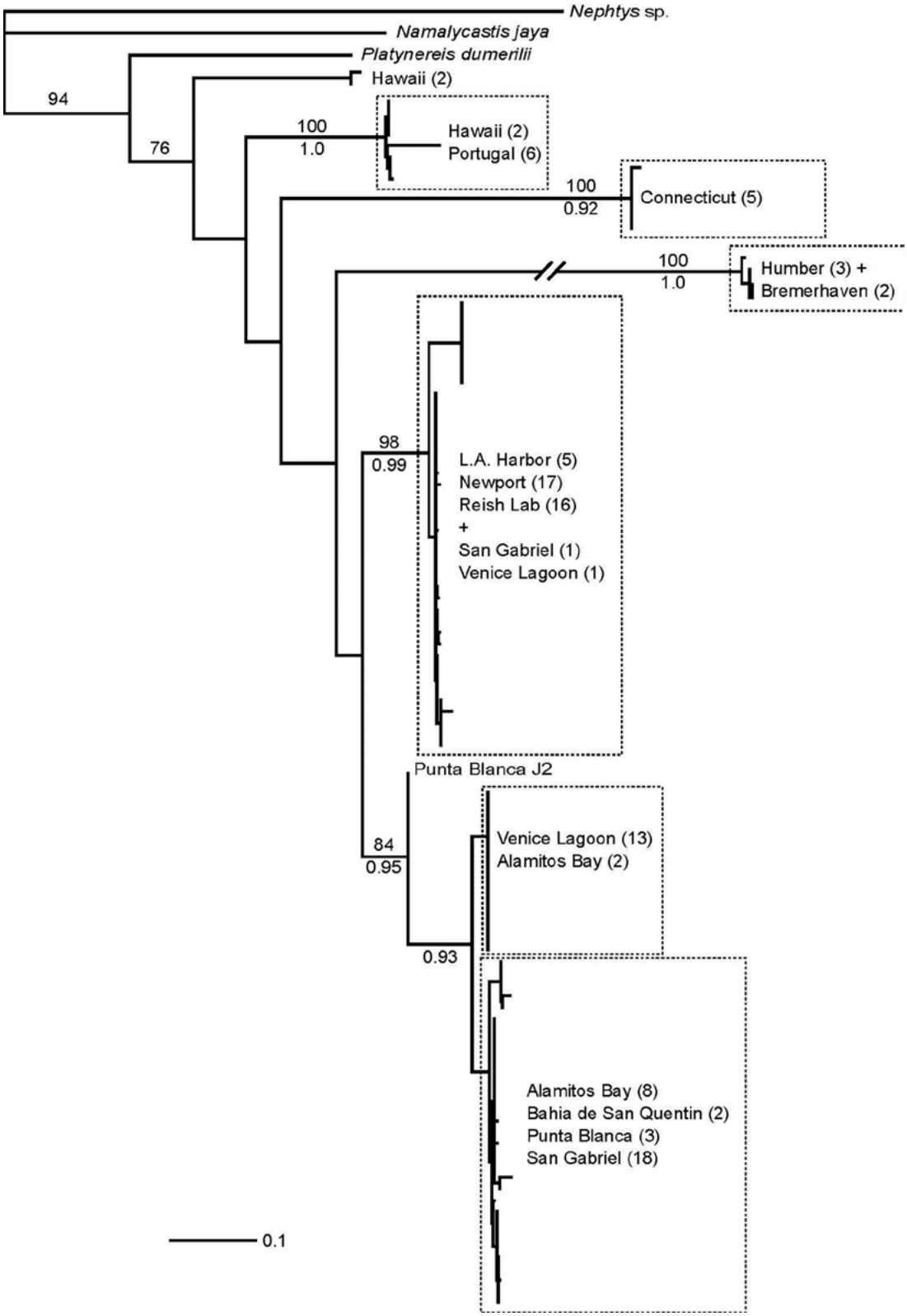

Figure 1. Maximum-likelihood tree resulting from partitioned analysis of the full concatenated data set in Garli 2.0. Numbers above branches represent ML bootstrap support values; numbers below branches represent posterior probabilities from an unpartitioned Bayesian analysis in MrBayes 3.2.1. Within each dashed box names refer to all populations present within that clade.

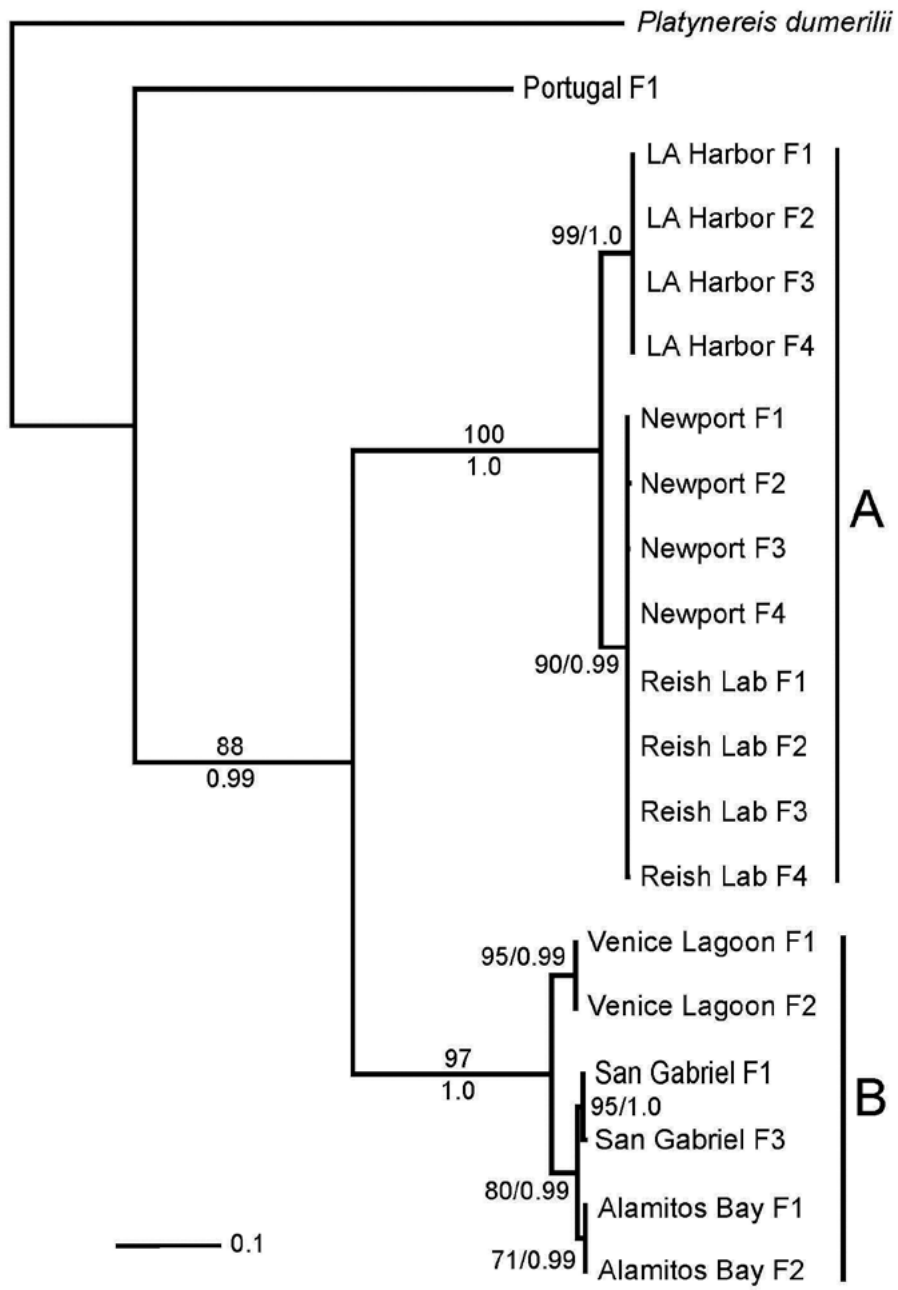

Figure 2. Maximum-likelihood tree resulting from partitioned analysis of the "all three genes" data set in Garli 2.0. Numbers above branches represent ML bootstrap support values; numbers below branches represent posterior probabilities from an unpartitioned Bayesian analysis in MrBayes 3.2.1

Table 3. Number of specimens (OTUs) and amount of missing data for the "full" and "all three genes" data matrices.

	Matrix	
	Full	**All three genes**
Number of OTUs	111	20
# outgroup OTUs	3[a]	1[b]
OTUs missing		
COI	37	0
16S	73	0
ITS	37	1[c]
OTUs with only:		
COI	21	0
16S	6	0
ITS	28	0
Total missing datad	56%	7.6%

a - *Nephtys* sp., *Namalycastis jaya* and *Platynereis dumerilii*
b - *Platynereis dumerilii* only
c - Missing from *Platynereis dumerilii*
d - includes terminal gaps, missing loci and indels in ITS

000001 (full) and 011110+F (all three genes), COI 3rd positions: HKY+G, 16S: TPM2uf+G (full) and 011212+G+F (all three genes), ITS1: K80+I (full) and 011010+G (all three genes) (substitution codes are from jModeltest; the TPM2uf model is the "three-parameter" or Kimura (1981) model, and has a substitution code of 010212). The best-fitting partitioning scheme for both the full and all-three-genes data set was the "five data subsets" scheme in which 16S, ITS1 and each COI codon position had a separate substitution model. Phylogenies resulting from maximum likelihood and Bayesian analyses of the "full" and "all three genes" data matrices under this partitioning scheme are presented in figures 1 and 2. Partitioned Bayesian analyses of the full data set failed to converge after >20 million generations; posterior probabilities shown in figure 1 are from an unpartitioned Bayesian analysis (GTR+I+G model) of that data set. The overall topologies of the trees are consistent with one another, with several well-supported nodes in both trees.

Sequences could not be obtained for all loci from every worm; in the full data matrix, 56% of the cells were missing data (including alignment gaps). In some cases, individual worms were represented by data from only one or two loci, resulting in nonoverlapping data among specimens. For example, for the four Hawaii specimens, we obtained only 16S data for two specimens, only ITS1 data from another specimen and both COI and ITS1 data (but not 16S) from a fourth specimen. The closest match to the two Hawaii 16S sequences was a 16S sequence from a Portugal specimen, resulting in a closer (but artifactual) relationship between these two Hawaii specimens and the other two in the data set (Fig. 1). Despite this, trees resulting from maximum likelihood and Bayesian analyses of individual-gene data sets (not shown) and the "full" and "all three genes" concatenated data sets were congruent, so we will focus on trees resulting from analyses of the full data matrix. The full matrix trees suggested that worms sampled from Connecticut, Hawaii and Portugal, as well as *N. diversicolor* Müller sampled from Germany and the UK, were genetically distinct from one another, and all analyses recovered a well-supported clade comprising all worms collected from Mexico and California (Fig. 1). This clade comprised two well-supported subclades, one consisting of worms collected from Los Angeles Harbour and Newport Beach (clade A), and the other consisting of worms sampled from all other southern California and Mexico (Punta Banda) population (clade B). Two members of Clade A (San Gabriel JDH 12 and Venice Lagoon JDH 1) were collected in localities generally inhabited by Clade B individuals.

Discussion

The finding that worms sampled from Connecticut, Hawaii, southern California, and Portugal form separate clades on our trees that correlate with geographic location is not particularly surprising. Some studies of polychaete species complexes with broad geographical distributions have yielded genetic evidence of substantial cryptic variation (e.g. *Neanthes diversicolor*; Virgilio et al., 2009), while others have revealed little genetic differentiation among widely separated regions (e.g., Ahrens et al., 2013). Unlike many polychaetes (but similar to *N. diversicolor*), species in the *Neanthes acuminata* complex have no pelagic larval stage. Species in this complex also use odour to initiate interpopulation aggression and pre-mating isolation. These life history features could partially explain why we seem to see genetic differences over short geographic distances. Worms sampled from Connecticut (2n=22) and Hawaii (2n=28) have different diploid chromosome numbers than do worms from California (2n=18), corroborating the inference that at least these three clades of worms in our phylogeny (Fig. 1) represent distinct species.

Two distinct subclades were found in southern California, one consisting of specimens collected from Los Angeles Harbour and Newport Beach (clade A), and the other comprising samples from all other southern California and Mexico sites (clade B). These clades were congruent with morphological and karyotypic differences seen among these populations—worms in clade A have red eyes and bright yellow/orange eggs, while worms in clade B have black eyes and pale yellow eggs. Since there are only two known populations in the *N. acuminata* complex with this colour distinction, we propose that the specimens from Los Angeles Harbour and Newport Bay were the result of a mutation giving rise to red eyes and bright orange ova. A similar eye colour mutation arose in a laboratory population of *Platynereis dumerilii* that was maintained for a long period of time at the Universität Köln (Fischer, 1969). The orange eye color was the result of a mutation that generated a recessive *or* allele. Backcrosses between black-eyed worms and the mutant form with red eyes produced a 1:1 ratio of black-eyed/orange-eyed

offspring. We speculate that a mutation producing the red eye/bright orange ova occurred in Newport Bay population which occurs intertidally in the back bay area. This mutant population may have been accidentally introduced into Los Angeles Harbour. It was a common practice for the owners of pleasure boats docked in Newport Bay to move their boats into the polluted waters of the inner harbour of the Los Angeles area to kill the fouling organisms attached to the vessel. Since *N. arenaceodentata* is known to live within the fouling organism community attached to boat floats (Crippen and Reish, 1969), the mutant could have been associated with such organisms attached to pleasure boats anchored in Newport Bay and were transported to Los Angeles Harbour in this way. The initial collection of *N. areaceodentata* was made in the west basin area of Los Angeles Harbour in December 1953 by DJR. This collection formed the basis of the life history study of the species (Reish, 1957). The ova were bright orange but the eye color was not noted. This population was destroyed prior to DJR moving to CSULB.

Additional evidence for the Newport-Los Angeles Harbour clade is the behavioural responses observed in the Southern California populations by Sutton et al. (2005). Black-eyed San Gabriel River worms showed more aggression toward red-eyed worms sampled from two sites (Newport and LA Harbour) than worms from the two red-eyed populations showed toward each other (though these findings were not statistically significant). Earlier Weinberg et al. (1992) reported that the inability of worms from the lab population to mate with worms from San Gabriel River and Newport Bay was evidence for rapid reproductive isolation of the lab worms following a founder event. However, this hypothesis was rejected by Rodriguez-Trelles et al. (1996) based on allozyme electrophoresis analyses of the three populations. Worms from the lab population and San Gabriel River produced offspring (DJR, personal observations). Worms collected from another locality in LA Harbour in 2008 by DJR were identical in appearance to the lab population, indicating little or no change from the 1964 collection.

The second clade on the Pacific Coast comprises worms from Venice, Alamitos Bay, San Gabriel River and Baja California. There are many estuaries in Southern California and Baja California and many of them have been altered in California, but those in Baja California have not been modified to any great extent. We assume that populations have existed in these areas for a long period of time. Historically, Alamitos Bay was an estuary formed by the San Gabriel River, but it became separated following a flood in 1938. The San Gabriel River became polluted and was devoid of benthic life by the late 1950s (Reish, 1956). Subsequently, the sources of pollution were eliminated and the channels were deepened. Shortly thereafter, electricity-generating plants were constructed and water was taken from Alamitos Bay for cooling the plants and discharged into San Gabriel River. *Neanthes arenaceodentata* was not found in San Gabriel River until 1971 (Reish unpublished report); we assume that they were introduced from Alamitos Bay.

Two members of Clade A (San Gabriel JDH 12 and Venice Lagoon JDH 1) were collected in localities generally inhabited by Clade B individuals, suggesting that limited migration occurred among these sites.

In conclusion, we have demonstrated that members of the *N. acuminata* complex sampled from multiple sites in the U.S., Mexico and Europe represent genetically distinct groups (possibly distinct species), and the different morphs of *N. arenaceodentata* seen in southern California represent two genetically distinct groups. We believe that the different populations seen in southern California may be the result of limited larval dispersion, the use of signature odour profiles for interpopulation aggression, pre-mating isolation, and preference for an estuarine habitat.

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