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Phylogeny and Genetic Diversity of Palolo Worms (*Palola,* Eunicidae) from the Tropical North Pacific and the Caribbean

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Abstract. Palolo worms (*Palola,* Eunicidae) are best known for their annual mass spawnings, or "risings," in the South Pacific. *Palola* currently contains 14 morphologically similar species, mostly from shallow tropical waters. In this study, 60 specimens of *Palola* from nine locations in the tropical North Pacific and the Caribbean were sequenced for the two mitochondrial markers cytochrome *c* oxidase subunit I and 16S ribosomal RNA to infer phylogenetic relationships, genetic diversity, and phylogeography within the taxon. Phylogenetic analysis was performed using Bayesian statistics and parsimony. Vouchers of the same specimens were examined morphologically. Two major clades (A and B) can be distinguished within the monophyletic *Palola.* A number of individuals in clade B bear rows of ventral eyespots in the posterior body region, typical for swarming *P. viridis* and probably a synapomorphy for clade B. No morphological synapomorphy was found for clade A. Haplotypes from divergent clades often co-occur in the same location. Some haplotypes are geographically widespread, in one case covering the entire east-west expansion of the tropical Pacific. These results imply that despite the apparent absence of teleplanic larvae in eunicid polychaetes, long-distance dispersal is possible in at least some lineages of *Palola.*

With the first taste of palolo I understood the Samoans' love for it. Certainly it suggested a salty caviar, but with something added, a strong, rich whiff of the mystery and fecundity of the ocean depths.

—R. Steinberg. *Pacific and Southeast Asian cooking.* Time-Life Books, New York, 1970

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Introduction

Early reports of swarming palolo worms and their use as a food source by the native populations of Samoa, Tonga, Fiji, and other South Pacific islands originated from European missionaries dispatched to these remote regions (*e.g.,* Codrington, 1891; MacDonald, 1858; Gray, 1847, cited in Stair, 1897). These authors were primarily interested in the anthropological aspects of the "rising," but the Reverend J.B. Stair provided the British Museum with specimens of the swarming stages from the Samoan islands, which Gray (1847) used as the basis for the species description of the Pacific palolo worm, *Palola viridis* (he chose a feminine ending for the genus name). Considering that the swarming stages were epitokes and no heads were present, this original description is very short. Friedlaender (1898, 1904) and Woodworth (1903, 1907) both retrieved complete worms and independently assigned them to *Eunice viridis.* The segments of the swarming epitokes of *Eunice viridis* each bear one dark pigment spot on the ventral surface. The pigment spots were examined histologically by Schröder (1905), who concluded that they were light sensors.

The common name, *palolo,* is based on the Samoan name for the worms. The Fijian name *mbalolo* and the Tongan name *balolo* are very similar. Other names are, or were, used in other swarming locations throughout the South Pacific (Table 1).

Later authors emphasized the regularity of the swarming, which is correlated with the lunar cycle and happens in October, November, or December in the South Pacific (Burrows, 1945, 1955; Caspers, 1961, 1984; Korringa, 1947). Annual risings of palolo worms have also been reported from the island of Ambon in Indonesia. In contrast to the South Pacific risings, in Ambon the event takes place in March or April. The worms are here known as *wawo* (Table

Table 1

Occurrences of Palola viridis *risings and common names where known*

Location	Local name	References
Samoa	palolo	Burrows (1945, 1955); Hauenschild *et al.* (1968); Woodworth (1903, 1907)
Fiji	mbalolo	Woodworth (1903); Burrows (1955)
Tonga	balolo	Burrows (1955)
Papua New Guinea	vaien, lamaha, kaama	Bartlett (1947); Burrows (1955)
Australia, east coast	—	Burrows (1955); Brown (1877)
Solomon Islands	orku, parenga or pareña	Burrows (1955)
Vanuatu	ayby (?), un	Burrows (1955); Codrington (1891); Seeman (1862)
New Caledonia	—	Burrows (1955)
Kiribati	Te nmatamata, te kawariki (?), te o (?)	Burrows (1955); Powell (1882)
Cook Islands	—	Burrows (1955)
Ambon	wawo	Burrows (1955); Horst (1904, 1905); Martens *et al.* (1995)

Dash (—) signifies that the local name is unknown.

1), but the swarming stages are actually a mix of 13 polychaete species (Martens *et al.* 1995). The wawo was identified as *Lysidice oele* by Horst (1904, 1905), but Martens *et al.* (1995) reported that the mix primarily contained *Palola viridis.*

Two other polychaetes have been described as "palolos." Both also form epitokes and swarm annually. The "Atlantic palolo" is *Eunice fucata.* Its swarming periodicity was described by Mayer (1908). The "Japanese palolo" is the brackish water nereidid *Tylorrhynchus heterochaetus,* which has been used as a model organism in physiology and embryology (*e.g.,* Osanai, 1978; Sato and Osanai, 1990). As neither is a *Palola,* I will not consider them further in this study.

Palola is morphologically characterized by the presence of two palps and three antennae, peristomial cirri, and scoop-shaped calcified mandibles, and by the absence of subacicular hooks (Fauchald, 1992). Branchial filaments, if present, are usually simple. Thus, many characters used in other eunicids to distinguish species, such as the shape and coloration of the subacicular hooks and the branching patterns of the branchial filaments, are not useful in *Palola.* Characters used by Fauchald in his review are mostly size ratios: length to maximum width of the specimen, relative length of antennae, palps, peristomial and notopodial cirri, as well as length ratios of appendages and shafts of compound falicigers. These differences are subtle and some of the type material was incomplete or poorly preserved, so Fauchald regarded his taxonomy as only a first step.

Three of the fourteen *Palola* species have wide geographic distributions (Table 2). *P. viridis* occurs all over the South Pacific, while *P. siciliensis* has been reported in all major oceans, roughly between latitudes 43 °N and 32 °S. *P. edentulum* might have a general subantarctic distribution. Apart from its type location on the Juan Fernandez Islands, it has also been reported from the North Island of New Zealand and the Chatham Islands in the Southwest Pacific and from the Magellanic Islands in the Southeast Pacific (Glasby and Alvarez, 1999). All other species are exclusively known from their type locations (Table 2).

Because morphological characters to distinguish species are limited and no information exists about intraspecific variation, I am here using a molecular approach to reconstruct the phylogeny within *Palola* and to assess genetic diversity and historical biogeography. Toward these goals, I sampled *Palola* species from the Caribbean and across the tropical North Pacific, sequenced them for two mitochondrial markers, and analyzed the sequence data in a phylogenetic and phylogeographic context.

Material and Methods

Collections

To retrieve specimens of *Palola* spp., *Eunice antennata, Eunice cariboea,* and *Dorvillea similis,* coral rubble was collected from seven Pacific and two Caribbean locations from depths varying from 0 to about 23 m, if necessary by snorkeling or scuba diving (see Appendix for collection information). Specimens were removed by breaking the rubble with hammer and chisel and pulling the worms out with forceps. In this process, most of the worms fragmented. In case of doubt whether two fragments belonged to the same individual, only one of the fragments (usually the one containing the head, or if no head was retrieved, the bigger fragment) was used. Usually, a small portion of each individual was fixed in 95%–100% ethanol for DNA studies. The remainder was treated as a voucher sample, fixed in 4% formalin in seawater, and later transferred to 70% ethanol. The voucher specimens are stored at the National Museum of Natural History in Washington, DC (USNM 1084310–USNM 1084406).

Table 2

Currently valid Palola *species and their type locations*

Species	Type location	Reported distribution*	References†
P. accrescens (Hoagland, 1920)	Philippine Islands	—	NA
P. brasiliensis Zanol *et al.,* 2000	Brazil	—	NA
P. ebranchiata (Quatrefages, 1866)	Palermo, Italy	—	NA
P. edentulum (Ehlers, 1901)	Juan Fernandez Island	South Australia, NZ North Island, Chatham Islands, Magellanic Islands	Glasby & Alvarez (1999)
P. esbelta Morgado & Amaral, 1981	São Sebastião, Brazil	—	NA
P. leucodon (Ehlers, 1901)	Juan Fernandez Island	—	NA
P. madeirensis Baird 1869	Madeira	—	NA
P. pallidus Hartman, 1938	Laguna Beach, California	—	NA
P. paloloides (Moore, 1904)	San Diego, California	—	NA
P. siciliensis (Grube, 1840)	Palermo, Italy	Mediterranean, SE USA, Mexico (Caribbean), Argentina, Venezuela, Galapagos Islands, Guam, South Australia, Thailand	Augener (1913); Gardiner (1976); Hofmann (1972, 1974, 1975); Kohn & Lloyd (1973); Kohn & White (1977); Liñero Arana (1985); Orensanz (1975); Salazar-Vallejo & Carrera-Parra (1997); Westheide (1977)
P. simplex Peters, 1854	Mozambique	—	NA
P. valida (Gravier, 1900)	Djibouti	—	NA
P. vernalis (Treadwell, 1922)	Fiji	—	NA
P. viridis Gray, 1847	Samoa	SW Pacific (s. Table 1)	s. Table 1

* Dashes (—) indicate that the species is known only from the type location.

† NA indicates that no further references exist after the species description.

Sequence generation

Total genomic DNA was extracted using a CTAB protocol (Thollesson, 2000) or a DNeasy kit (Qiagen). Gene regions of the mitochondrial genes for large subunit ribosomal RNA (16S rRNA) and for cytochrome *c* oxidase subunit I (COI) were amplified by polymerase chain reaction (PCR). PCR reactions were performed in a volume of 25 or 50 μl. For each 25-μl reaction, 5–10 ng DNA (CTAB protocol) or 1 μl of the extractions (Qiagen protocol) and 0.625 units of taq polymerase (Promega) were used. The concentration of other reagents were 200 μM each of dATP, dGTP, dCTP, and dTTC, 0.5–1 μM of each primer, and 1X sequencing buffer. If amplification was unsuccessful even at lower annealing temperatures, it could often be achieved using the MasterTaq kit (Eppendorf), according to the manufacturer's instructions. The following primers were employed: 16Sa (5′-CGCCTGTTTATCAAAAACAT-3′ [Xiong and Kocher, 1991]) and 16Sbr (5′-CCGGTCTGAACTCACATCACGT-3′ [Palumbi, 1996]) for 16S rRNA; the primer pairs LCO (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO (5′-TAAACT TCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.,* 1994) and COI-7 (5′-ACNAAYCAYAARGAYATYGGNAC-3′) and COI-D (5′-TCNGGRTGNCCRAANARYCARAA-3′) (Saito *et al.,* 2000) in all possible combinations for COI. PCRs were performed according to standard protocols with annealing temperatures of 42° to 45° C. PCR products were visualized in 1%–1.5% agarose gels stained in ethidium bromide and cleaned using the GENECLEAN II kit (Bio 101) or the QIAquick PCR purification kit (Qiagen).

Sequence reactions were performed in 10-μl volume, using 1 μl (if cleaned with GENECLEAN) or 5 μl (if cleaned with QIAquick) of the sample, 1 μM of primer, 2 μl of ABI BigDye Terminator ver. 3.1 (Applied Biosystems) and 2 μl halfTERM Dye Terminator reagent (Genpak).

Sequence reactions were performed with the same thermal cycler as for PCR reactions, using standard protocols. After cleanup of the sequence reactions using gel filtration cartridges from Edge Biosystems, the sequences were analyzed with an ABI 377 or 3100 automatic sequencer. Electrochromatograms from the sequencer were visualized in Sequencher 4.0. Forward and reverse fragments were assembled and primer regions cropped and discarded. Outgroup sequences for *Marphysa belli, Marphysa sanguinea, Ophryotrocha gracilis, Lumbrineris funchalensis,* and *Hyalinoecia tubicola* were from Struck *et al.* (2005), Dahlgren *et al.* (2001), and Siddall *et al.* (2001). All sequences have been deposited in GenBank with accession numbers DQ317807 to DQ317917 (Table 3).

Table 3

Collection information and COI and 16S GenBank accession numbers for Palola *samples*

Sample name*	Station	COI accession number†	16S accession number†
Belize14	1A	DQ317809	DQ317863
Belize32	1B	DQ317810	DQ317864
Belize34	1B		DQ317865
Belize37	1C	DQ317811	DQ317866
Belize38	1C	DQ317812	
Belize39	1C	DQ317813	DQ317867
Belize43	1B		DQ317868
Bocas68	3A	DQ317814	DQ317869
Bocas70	3A	DQ317815	DQ317870
Bocas77	3A	DQ317816	DQ317871
Bocas78	3B	DQ317817	DQ317872
Bocas79	3B	DQ317818	DQ317873
Bocas85	3A		DQ317874
Bocas86	3C		DQ317875
Bocas87	3C		DQ317876
Perlas52	4	DQ317838	DQ317897
Perlas53	4	DQ317839	
Perlas54	4	DQ317840	DQ317898
Perlas55	4	DQ317841	DQ317899
Perlas57	4	DQ317842	DQ317900
Perlas58	4	DQ317843	
Perlas59	4	DQ317844	DQ317901
Perlas61	4	DQ317845	DQ317902
Perlas63	4	DQ317846	DQ317903
Guam89	5A	DQ317823	DQ317881
Guam92	5B	DQ317824	DQ317882
Guam94	5C	DQ317825	DQ317883
Guam100	5C	DQ317819	DQ317877
Guam101	5C	DQ317820	DQ317878
Guam102	5D	DQ317821	DQ317879
Guam103	5D	DQ317822	DQ317880
Palau105	6A	DQ317831	
Palau111	6A	DQ317832	DQ317891
Palau115	6B	DQ317833	DQ317892
Palau117	6B	DQ317834	DQ317893
Palau118	6C	DQ317835	DQ317894
Palau124	6D	DQ317836	DQ317895
Palau125	6D	DQ317847	DQ317896
Yap129	7A	DQ317852	DQ317911
Yap130	7A	DQ317853	DQ317912
Yap131	7A	DQ317854	DQ317913
Yap138	7A	DQ317855	
Yap141	7B	DQ317856	DQ317914
Pohnpei142-1	8A	DQ317847	DQ317904
Pohnpei142-2	8A		DQ317905
Pohnpei151-1	8B		DQ317906
Pohnpei151-2	8B	DQ317848	DQ317907
Pohnpei151-3	8B	DQ317849	DQ317908
Pohnpei157-1	8C	DQ317850	DQ317909
Pohnpei157-2	8C	DQ317851	DQ317910
Ant158-1	9	DQ317807	DQ317860
Ant158-3	9		DQ317861
Ant160	9	DQ317808	DQ317862
Kosrae161	10A	DQ317826	DQ317884
Kosrae165	10B	DQ317827	DQ317885
Kosrae166	10B	DQ317828	DQ317886
Kosrae168	10B	DQ317829	DQ317887
Kosrae169	10B		DQ317888
Kosrae170	10B		DQ317889
Kosrae176	10C	DQ317830	DQ317890
Eunice antennata	1D	DQ317858	DQ317916
Eunice cariboea	1E	DQ317859	DQ317917
Marphysa belli			AY838835
Marphysa sanguinea		AY040708.1	AY838836
Dorvillea similis		DQ317857	DQ317915
Ophryotrocha gracilis			AF321424
Lumbrineris funchalensis			AY838831
Hyalonoecia tubicola			AY838830

* Sample names for Palola refer to the collecting locations, followed by individual identifiers that refer to the vials of the voucher material; sample names with dashes refer to several specimens from the same vial. The outgroups are specified by their full species names.

† Empty cells indicate that no sequence was obtained.

Analysis

The alignment of the COI sequences produced no ambiguities. The ribosomal sequences were submitted to the MAFFT server in Kyoto (http://www.biophys.kyoto-u.ac.jp/webmafft/ [Katoh *et al.,* 2002]) for complete alignment. In addition to the taxa included in this study, the alignment also included the sequence for the chiton *Katharina tunicata* 2 (GenBank accession code U09810), downloaded with secondary structure annotations from the European ribosomal database (http://www.psb.ugent.be/rRNA/ [Van de Peer *et al.,* 2000]). The hypervariable loop between the stem regions G3 and G3′ (positions 247–288 in the alignment) was excluded from the phylogenetic analysis. The analysis files with the aligned sequences have been deposited with Tree BASE and are available through the World Wide Web at http://www.treebase.org.

Phylogenetic analysis was performed using Bayesian statistics in MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) and parsimony analysis in PAUP* (Swofford, 2003). The genes were analyzed separately and in combination.

For the stem regions of the 16S rRNA sequences, a list of nucleotide pairings was assembled manually using the annotated sequence of *Katharina tunicata* as a reference for secondary structure. The stem regions were analyzed under a doublet model with a single rate parameter and 16 states (Schöninger and von Haeseler, 1994), representing all possible nucleotide pairings. The 16S loop regions and the COI sequences were separately submitted to MrModeltest 2.2 (Nylander, 2004), which tests 24 models of sequence evolution using four hierarchical likelihood ratio tests (hLRTs) and the Akaike information criterion. For COI, all four hLRTs as well as the Akaike information criterion favored

a general time reversible model (Tavaré, 1986) with a correction for a gamma distribution of substitution rates and a proportion of invariable sites (GTR+G+I). For the 16S loop regions, the Akaike information criterion and two of the likelihood ratio tests (hLRT2 and hLRT4) favored an HKY+I+G model (Hasegawa *et al.,* 1985); the default hLRT1 favored a GTR+G+I model; hLRT3 favored GTR+G. As it is the more general model, GTR+I+G was implemented for both gene regions. *Dorvillea similis* was set as the outgroup. For the Bayesian analyses, two runs, with four Monte Carlo Markov chains each, were performed simultaneously for 5,000,000 generations each, sampling trees every 500 generations. The temperature parameter was set to 0.07. The initial 2,500,000 trees from each run were discarded as "burn-in." The remaining 5000 trees from each run were combined and summarized in a majority rule consensus tree.

Parsimony bootstrap analysis was performed with 1000 bootstrap replicates, using the heuristic search option in PAUP*. For each heuristic search, 10 replicates of random taxon addition were performed with tree bisection/reconnection as the branch-swapping algorithm. Branches with less than 50% bootstrap support were discarded.

Haplotypes were collapsed using the program Collapse 1.2 (Posada, 2004). Uncorrected genetic distances were calculated in PAUP* (Swofford, 2003). Molecular diversity indices were calculated in Arlequin 2.000 (Schneider *et al.,* 2000). Nucleotide diversity was calculated under the Kimura 2-parameter model. Geographic surface distances between sample locations were calculated using the airports of the respective locations as reference points—with the exception of Ant Atoll, for which the position was obtained from the website of the U.S. Geological Survey, and Carrie Bow Cay in Belize, for which the location was obtained from the CCRE program, Smithsonian Institution.

Results

Phylogenetic analysis

For 16S rRNA, sequence length varied between 362 bp and 509 bp. These differerences are based on complete sequences, that is, not on sequences with missing end regions. For five of the eight outgroups (*Hyalonoecia tubicola, Lumbrineris funchalensis, Ophryotrocha gracilis, Marphysa belli,* and *Marphysa sanguinea*), the 16S sequences were markedly shorter than all others. The alignment with secondary structure annotations indicates that these five species are missing entire stem/loop regions: the complementary strands of G3/G3′, G8/G8′, G9/G9′, and G15/G15′ are absent, as are the loop regions between the respective pairs. The region that aligns with the G7 region in other species is partially present but does not seem to have a complement.

In the combined analysis there was no conflict between the Bayesian and the bootstrap parsimony analysis, but the Bayesian analysis gave better resolution in some parts of the tree, especially in the deeper nodes. *Palola* appeared as monophyletic, with 99% posterior probability and 69% bootstrap support (Fig. 1). The sister group to *Palola* remains unresolved. Within *Palola,* two major clades can be distinguished: clade A contains eastern and western Pacific samples, plus one sample from Bocas del Toro in the Caribbean. Clade B contains a mix of Caribbean and western Pacific samples. The clades are further subdivided into nine subclades each, A1–A9 and B1–B9, respectively.

When both genes were analyzed separately (Fig. 2), all subclades remained supported, with one exception: there was no support for clade A2 in the Bayesian analysis of 16S (however, the clade had 70% parsimony bootstrap support). There are some discrepancies between the separate analyses and the combined analysis, but in all cases of discrepancies the alternative arrangement to the combined analysis has low support, both in posterior probabilities and bootstrap values. The 16S rRNA analysis supports the monophyly of *Palola* and resolves clade B. The COI analysis gives low resolution in the deeper nodes but resolves clade A.

Morphological observations

Due to the incompleteness of much of the material and the high degree of morphological conservation within the genus, the clades shown in Figure 1 cannot be clearly delineated morphologically. However, in some cases characters are restricted to certain clades, although they were not necessarily observed in every individual. In particular, in clade B, seven individuals (marked with asterisks in Fig. 1) showed ventral eyespots in their posterior body regions (Fig. 3A). In no case have ventral eyespots been observed in clade A. Clade A8 contains three individuals that were unusual with respect to morphology and habitat: they have unusually long and tapering antennae, palps, and parapodial cirri (Fig. 3B); and unlike all other samples, they were not infaunal, but inhabited crevices under rocks. Another morphologically distinct clade is clade A7, containing two specimens from Pohnpei. These are characterized by dark brown pigment on the dorsal side; in other samples, body pigmentation is usually restricted to the ventral eyes and the parapodial pigment spots. In addition, the two specimens have a median sulcus in the prostomium that is markedly shallow and barely visible dorsally (Fig. 3C).

Haplotype diversity and distribution

The 50 COI sequences grouped into 26 haplotypes; the 55 16S sequences grouped into 32 haplotypes. Number and percentage of singleton (only found in a single individual), private (occurring in more than one individual but a single location), and shared haplotypes (occurring in more than one location) are listed in Table 4.

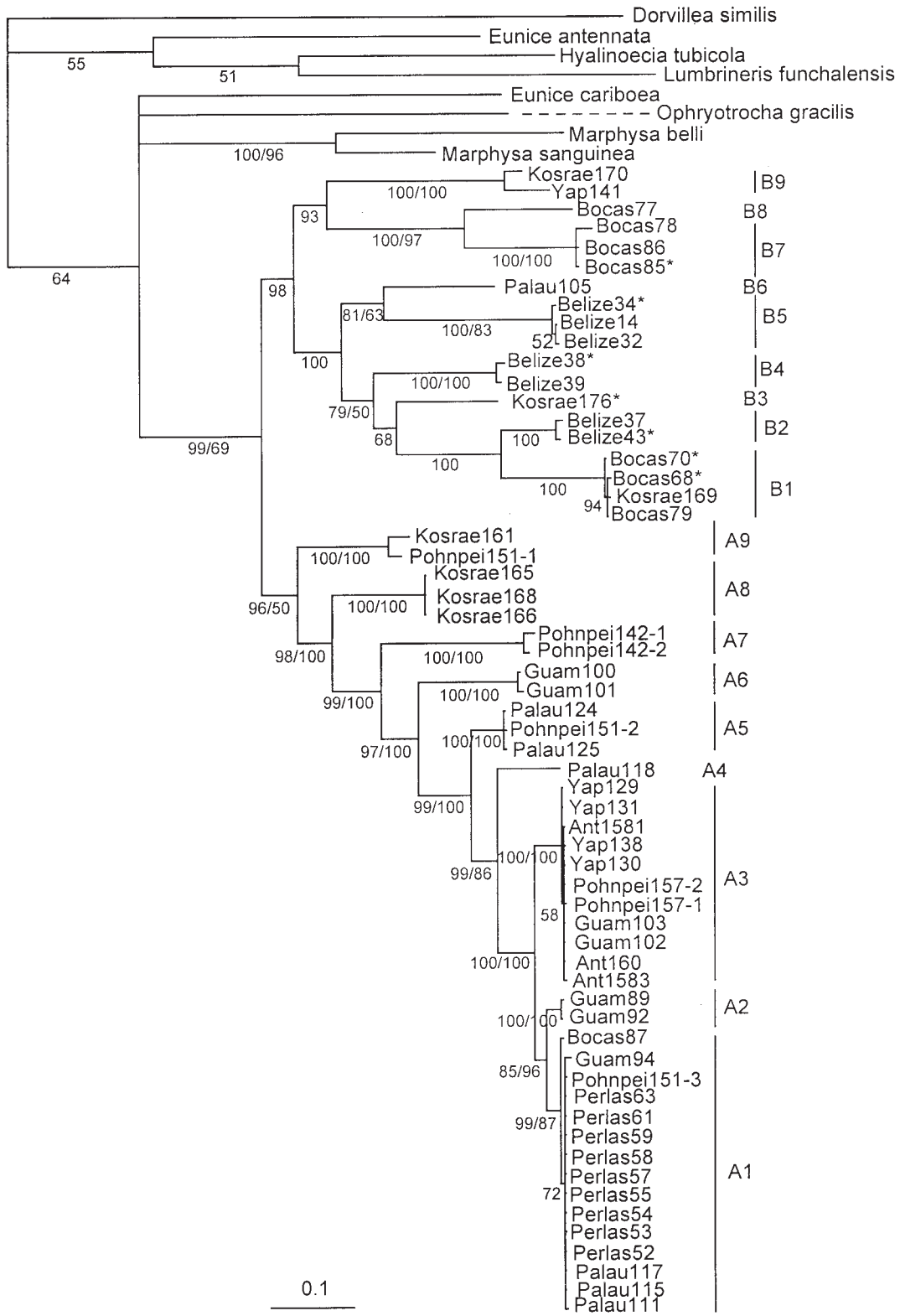

Figure 1. Bayesian analysis, 50% majority rule consensus from combined 16S ribosomal RNA and cytochrome oxidase subunit I. All *Palola* are named after their geographic origin, followed by a unique identifier as in Table 3. Asterisks indicate samples with ventral eyespots. Names of outgroup species are given in full. Branch support: posterior probability/parsimony bootstrap (single numbers indicate posterior probabilities at branches for which the bootstrap percentages were $< 50\%$).

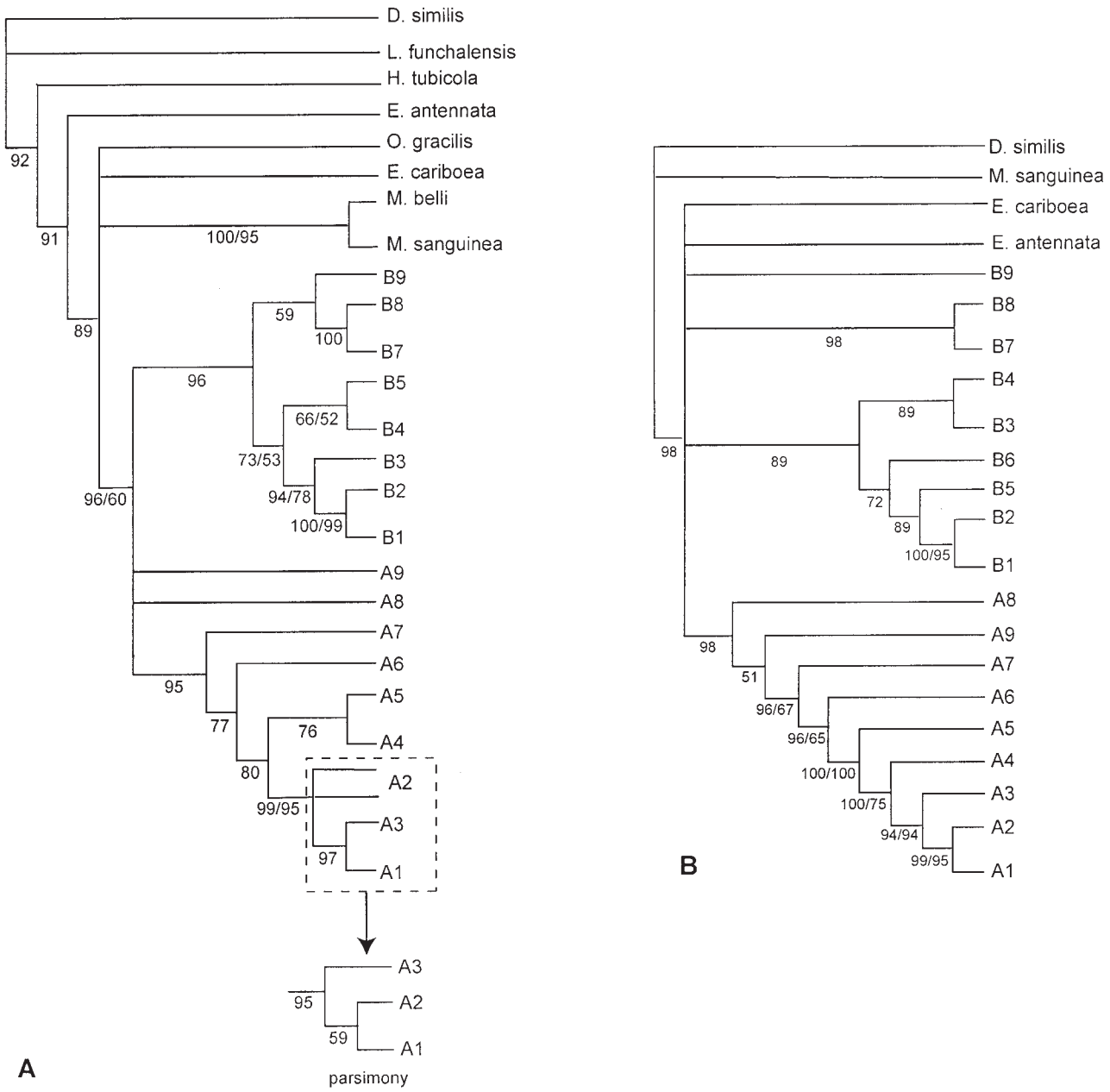

Figure 2. Cladograms resulting from separate analyses for both genes, Bayesian analysis, 50% majority rule consensus trees. With the exception of clade A2 in the analysis of 16S rRNA, all subclades remained supported in the separate Bayesian analyses. In both trees, clades A1–A9 and B1–B9 correspond to clade designations in Figure 4. Branch support: posterior probability/parsimony bootstrap (single numbers indicate posterior probabilities at branches for which the bootstrap percentages were $< 50\%$). (A) Results of 16S rRNA analysis. In the parsimony analysis, A2 is monophyletic with 70% bootstrap support and appears as the sister group to clade A1 (inset). (B) Results of COI analysis.

No haplotype or nucleotide diversity was found in Ant Atoll or in Las Perlas for either COI or 16S, indicating that all individuals from these locations were identical (however, only three individuals were sequenced from Ant Atoll). The highest haplotype diversity (1 for both genes) was in Pohnpei, where each sampled individual represented a different haplotype. Haplotype diversity was also high in each of the remaining locations (Table 5). Nucleotide diversity was higher in the Caribbean samples than in any of the Pacific samples for both of the genes. Mean nucleotide divergences among and within clades are listed in Table 6.

All shared haplotypes spanned distances of over 2000 km

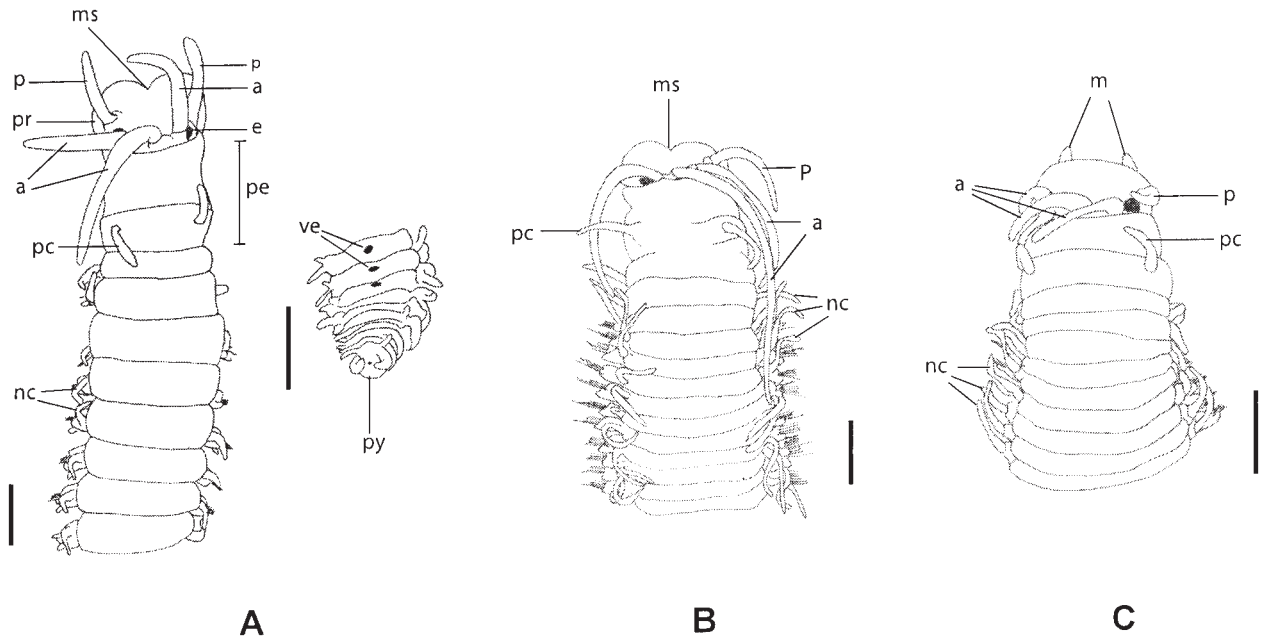

Figure 3. Morphological diversity in *Palola:* a, antenna; e, eye; m, mandible; ms, median sulcus of prostomium; nc, notopodial cirrus; p, palp; pe, peristomium; pc, peristomial cirrus; pr, prostomium; py, pygidium; ve, ventral eyespots. (A) *Palola* sp. (Bocas85). Left: anterior, right: posterior end with ventral eyespots. The eyespots extend over more setigers than shown; scale bars = 500 μm. (B) *Palola* sp. (Kosrae165); note long palp (left palp missing), antennae, peristomial and notopodial cirri; scale bar = 1 mm. (C) *Palola* sp. (Pohnpei 142-1); note reduced median sulcus of prostomium; scale bar = 1 mm.

(Tables 7, 8). The most widespread haplotypes covered the complete east-west expansion of the tropical North Pacific, from Las Perlas in the east to Palau in the west (15,826 km). Private haplotypes occurred in Belize, Bocas, Kosrae, and Yap.

Discussion

Despite a high degree of morphological uniformity, the phylogenetic analyses reveal deep genetic divergences within *Palola.* 16S rRNA is an appropriate marker to resolve the deeper branches within the genus (Fig. 2A), but even more conserved genes, such as the nuclear 18S rRNA, might be necessary to resolve outgroup relationships. Cytochrome *c* oxidase subunit I (COI) provides better resolution than 16S rRNA within the more derived groups in clade A (Fig. 2B), but faster evolving markers would be necessary to address some phylogeographic questions.

Morphological examinations of the voucher material revealed that few morphological characters distinguish the clades. The only likely morphological distinction between clades A and B are the ventral eyespots, as described in *Palola viridis.* They are only present in some individuals of clade B but occur in five of the nine subclades. One obvious explanation for why they were not present in every specimen is that often only anterior fragments were collected and examined, the posterior body regions being missing. Another explanation is that eyespots only develop during reproductive time. Schröder (1905) examined the ventral eyespots of *P. viridis* histologically, using the swarming epitokes, but never studied nonreproductive individuals. It is a strong possibility that eyespots are a synapomorphy for clade B. Reproductive specimens in clade A never had ventral eyespots. No morphological synapomorphy for clade A has been detected.

At present, it is impossible to determine which, if any, of

Table 4

Number and percentages of singleton, private and shared haplotypes for COI and 16S rRNA

	Haplotypes		Individuals	
	Number	%	Number	%
Cytochrome *c* oxidase subunit I				
Singletons	20	76.9	20	40
Privates	4	15.4	10	20
Shared	2	7.7	20	40
Total	26		50	
16S rRNA				
Singletons	24	75.0	24	43.6
Privates	4	12.5	9	16.4
Shared	4	12.5	22	40.0
Total	32		55	

Table 5

Haplotype (h) *and nucleotide diversity* (π) *for both genes by location*

	COI		16S	
	Haplotype diversity (h)	Nucleotide diversity (π)	Haplotype diversity (h)	Nucleotide diversity (π)
Belize	0.900 ± 0.161	0.143 ± 0.087	0.933 ± 0.122	0.108 ± 0.063
Bocas	0.700 ± 0.218	0.164 ± 0.099	0.964 ± 0.077	0.165 ± 0.090
Palau	0.952 ± 0.095	0.115 ± 0.065	0.867 ± 0.129	0.052 ± 0.031
Yap	0.900 ± 0.161	0.087 ± 0.053	0.833 ± 0.222	0.102 ± 0.067
Guam	0.900 ± 0.095	0.113 ± 0.064	0.952 ± 0.095	0.059 ± 0.034
Pohnpei	1.000 ± 0.126	0.126 ± 0.077	1.000 ± 0.076	0.099 ± 0.056
Ant	0	0	0	0
Kosrae	0.700 ± 0.218	0.117 ± 0.072	0.857 ± 0.137	0.136 ± 0.077
Perlas	0	0	0	0

the subclades in clade B is *P. viridis.* All of the subclades are morphologically similar and, whenever relevant characters were observable, more or less conform to the description of *P. viridis* given in Fauchald (1992): palps and antennae are arranged in a horseshoe; the size of the head appendages increases from the palps to the median antenna; the prostomium is about as wide as the peristomium; the prostomium in some specimens is dorsally excavate around the palps and the lateral antennae. Characters of the parapodia and setae widely overlap with the descriptions of other species and have limited value for species identification. Fauchald (1992) describes brown pigmentation in the anterior dorsum of *P. viridis* that was not observed in any samples belonging to clade B. Because of the uncertainties associated with designating any of the subclades of clade B as *P. viridis,* it is desirable to generate DNA sequences for samples originating from the type location of this species in Samoa and throughout the species' geographic range to determine its true distribution and genetic diversity. Attempts to obtain material from Samoa have so far been unsuccessful.

Table 6

Mean nucleotide divergence and range (in parentheses) in COI (uncorrected) and 16S rRNA (uncorrected) within and between clades for individuals and haplotypes

	Individuals (%)	Haplotypes (%)
Cytochrome *c* oxidase subunit I		
Within *Palola*	14.5 (0–24.2)	17.1 (0.2–24.2)
Within clade A	9.8 (0–20.0)	13.2 (0.2–19.7)
Within clade B	16.6 (0–21.4)	17.4 (0.1–21.2)
Between clades A and B	20.7 (14.6–24.3)	20.0 (14.7–24.2)
16S rRNA		
Within *Palola*	12.4 (0–21.9)	14.6 (0.2–21.9)
Within clade A	6.4 (0–13.9)	9.2 (0.2–18.8)
Within clade B	15.3 (0–21.9)	15.4 (0.2–21.9)
Between clades A and B	18.2 (3.1–21.8)	18.0 (3.6–21.8)

No clear morphological distinction is apparent among the closely related clades A1, A2, and A3. They may represent a single species with a distribution across the entire east-west expansion of the Pacific and even the Caribbean. If the

Table 7

List of shared (COI-S1 and COI-S2) and private haplotypes (COI-P1 through COI-P4) for COI with geographic extensions of shared haplotypes

COI Haplotype name	Samples	Maximum surface distance (km)
CO1-S1	Ant158-1	2,202
	Ant160	
	Guam102	
	Guam103	
	Pohnpei157-2	
	Yap130	
	Yap138	
CO1-S2	Guam94	15,826
	Palau115	
	Palau117	
	Pohnpei151-3	
	Perlas52	
	Perlas53	
	Perlas54	
	Perlas55	
	Perlas57	
	Perlas58	
	Perlas59	
	Perlas61	
	Perlas63	
CO1-P1	Belize14	
	Belize32	
CO1-P2	Bocas68	
	Bocas70	
	Bocas79	
CO1-P3	Kosrae165	
	Kosrae166	
	Kosrae168	
CO1-P4	Yap129	
	Yap131	

Table 8

List of shared (16S-S1 through 16S-S4) and private haplotypes (16S-P1 through 16S-P4) for 16S rRNA with geographic extensions of shared haplotypes

16S Haplotype name	Samples	Maximum geographic distance (km)
16S-S1	Ant158-1	2,202
	Ant158-3	
	Ant160	
	Guam102	
	Guam103	
	Pohnpei157-1	
	Pohnpei157-2	
	Yap130	
16S-S2	Palau115	15,826
	Palau117	
	Perlas52	
	Perlas54	
	Perlas55	
	Perlas57	
	Perlas59	
	Perlas61	
	Perlas63	
16S-S3	Bocas79	8,888
	Kosrae169	
16S-S4	Palau124	7,406
	Palau125	
	Pohnpei151-2	
16S-P1	Belize14	
	Belize34	
16S-P2	Bocas85	
	Bocas86	
16S-P3	Kosrae165	
	Kosrae166	
	Kosrae168	
16S-P4	Yap129	
	Yap131	

identification key in Fauchald (1992) is used, the species is identified as *P. siciliensis*; however, the specimens can be distinguished from *P. siciliensis* by their earlier onset of branchiae. In clades A1–A3, branchiae start between setigers 48 and 60 whenever a long enough anterior fragment can be observed. In *P. siciliensis,* the earliest appearance of branchiae is setiger 92 and can be as far posterior as setiger 180 (Fauchald, 1992). As with *P. viridis, P. siciliensis* lacks sequence data from the type location in Sicily to test the species designation.

All subclades except A1–A3 may represent distinct species. The species generally seem to be cryptic, although clades A7 and A8 show some characteristic morphological features (Fig. 3) not observed in other specimens or described from any of the known species. However, for clearer morphological delineations, it would be necessary to examine more complete material for all subclades.

The age of *Palola* as a genus is unknown, but the probability is high that it arose in the Paleozoic. Fossil mandibles and maxillae of eunicidan polychaetes date back to the Ordovician (Kielan-Jaworowska, 1966), including the lapidognath jaw type found in the Eunicidae. However, the typical scoop-shaped mandibles of *Palola* have not been described from the fossil record. A paleozoic origin would explain the high degree of intrageneric genetic divergence.

Although COI and 16S rRNA are considered two of the more conserved genes in the mitochondrial genome, the relatively high degree of divergence—averaging 14.5% and 12.4% respectively—is not unusual in polychaetes. For 16S rRNA, mean sequence divergence in the syllid genus *Autolytus* is approximately 21% (Nygren and Sundberg, 2003), based on 16 species. For the dorvilleid genus *Ophrytrocha* it is 12%, based on 17 species (Dahlgren *et al.,* 2001). For COI, mean within-family divergence in the Terebellidae is over 20% based on the nine available sequences from GenBank (Colgan *et al.,* 2001; Siddall *et al.,* 2001). For the two terebellid genera of which two species are represented in GenBank, sequence divergence was 20% for the two *Loimia* species and 19% for the two *Amphitrite* species. In view of the fact that taxonomic ranks are arbitrary, these comparisons can be only a rough guide, but they convey that the genetic variation within *Palola* is within a normal range for polychaetes. The genetic variation is only surprising compared with the high degree of morphological conservation among the species.

Most haplotypes (76.9% for COI; 75% for 16S rRNA) detected in this study are singletons (Table 4) and uninformative with respect to phylogeographic questions. Only a small percentage of the haplotypes (7.7% for COI; 12.5% for 16S) are shared among locations, but they represent a large percentage of the sampled individuals (40% for both markers) and cover six of the nine collecting locations (Tables 7 and 8). Several recent studies have shown strong geographic population structure in marine shallow-water invertebrates, even in taxa with high dispersal capabilities (*e.g.,* Kirkendale and Meyer, 2004, and references therein). It is therefore surprising to find widespread, in some cases extremely widespread, haplotypes and clades in *Palola,* especially considering that all known eunicid larvae are short-lived and lecithotrophic (Richards, 1967). In clades A1–A3 with very short branches, the lack of geographic structure may be due to incomplete lineage sorting, suggesting that the islands have been colonized relatively recently in terms of the age of the genus and not enough time has passed for distinct lineages to be established on each island (*e.g.,* Harrison, 1991; Avise, 2000).

Haplotype diversity and nucleotide diversity are both high in all but two collecting locations (Table 5). The phylogenetic reconstructions show that the high nucleotide diversity is not due to local radiations (in which case all haplotypes in one location would form a clade) but to repeated colonization of the islands by members of genetically divergent *Palola* clades. This effect is most pro-

nounced in the Caribbean locations and is probably related to the complex geological history of the Caribbean region. Multiple models exist for the tectonic history of the Caribbean (Graham, 2003, and references therein), but whichever theory is favored, it is likely that shallow-water marine taxa such as *Palola* have had numerous opportunities for dispersal and vicariance within the region, allowing divergent clades to co-occur within the same location. Exchange with Pacific waters was possible until approximately 3.5 million years ago, explaining why the two major *Palola* clades contain samples from both the Caribbean and the Pacific.

No nucleotide and haplotype diversity was detected in Ant Atoll or the Las Perlas Archipelago. Although this might be an artifact of small sample size (only three individuals sampled from Ant Atoll) or a single collecting spot at each island group, it could also indicate that these locations were only recently colonized. If a population had been established for a long time, some local haplotype diversity would be expected. More extensive sampling would be necessary to investigate this question.

More rapidly evolving markers should add resolution to clade A, but the current data indicate that long-distance dispersal has taken place repeatedly in both major clades of *Palola.* Despite long-held opinions on eunicid development, long-lived planktotrophic larvae might exist in at least some *Palola* lineages.

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Appendix

Collecting Stations: Geographic co-ordinates refer to airports, with the exception of Ant Atoll and Carrie Bow Cay

Station 1: Carrie Bow Cay area, Belize (16 °48′N, 88 °05′W)
- 1A: Carrie Bow Cay, reef flat, 0.5–1 m, Feb. 2001
- 1B: Carrie Bow Cay, reef drop-off, 8–10 m, Feb. 2001
- 1C: Blue Ground Range, 0.5–1 m, Feb. 2001
- 1D: Twin Cays, 0.5–1 m, Feb. 2001
- 1E: Southwater Cay, 1.5 m

Station 2: Saboga Island, Las Perlas Archipelago, Panama (8 °35′N, 79 °35′W): 2.5–3 m, June 2001

Station 3: Bocas del Toro Archipelago, Panama (9 °21′N, 82 °15′W)
- 3A: Hospital Point, Isla Solarte, 2.5 m, June 2001
- 3B: Mangrove Inn, Isla Colón, 1–2 m, June 2001
- 3C: Drago Beach, Isla Colón, Panama, 2 m, June 2001

Station 4: Guam, USA (13 °29′N, 144 °47′E)
- 4A: Double Reef, 15–20 m, Oct. 2001
- 4B: Shark's Pit, 15–23 m, Oct. 2001
- 4C: Cocos Island, 1.5–3 m, Oct. 2001
- 4D: Mangilao, 1 m, Oct. 2001

Station 5: Republic of Palau (7 °22′N, 134 °32′E)
- 5A: Lighthouse Reef, 1.5–2 m, Oct. 2001
- 5B: Western Channel, 2 m, Oct. 2001
- 5C: Short drop-off, 13 m, Oct. 2001
- 5D: Turtle Island, 0.5–2 m, Oct. 2001
- 5E: Ngerikuul Channel, 13–17 m, Oct. 2001

Station 6: Yap, Federated States of Micronesia (9 °29′N, 138 °40′E)
- 6A: Colonia, 3–5 m, Nov. 2001
- 6B: Mill Channel, 5–15 m, Nov. 2001

Station 7: Pohnpei, Federated States of Micronesia (6 °59′N, 158 °12′E)
- 7A: The Village Hotel, 0.3–1.5 m, Nov. 2001
- 7B: Nahpali, 2 m, Nov. 2001
- 7C: Black Coral Island, 1.5–3 m, Nov. 2001

Station 8: Tolonmurui Island, Ant Atoll, Pohnpei, Federated States of Micronesia (6 °46′N, 157 °55′E), 0.1–5 m, Nov. 2001

Station 9: Kosrae, Federated States of Micronesia (5 °21′N, 162 °57′E)
- 9A: Mwot, Kosrae, Federated States of Micronesia, 2–3 m, Nov. 2001
- 9B: Buoy 21, Kosrae, Federated States of Micronesia, 13–20 m, Nov. 2001
- 9C: Buoy 5, Kosrae, Federated States of Micronesia, 10–15 m, Nov. 2001