



Sister-species of eastern Pacific hydrothermal vent worms (Ampharetidae, Alvinellidae, Vestimentifera) provide new mitochondrial *COI* clock calibration

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Introduction

The evolutionary age of some of the hydrothermal vent taxa have recently become a highly debated issue. Vestimentiferan worms (= siboglinid polychaetes) in particular, have been claimed to be as old as 430 million years based on the occurrence of fossilized tubes resembling those of currently known Vestimentifera (Little et al., 1997), while other authors, based on molecular data, have claimed they must be younger than 100 million years old (Black et al., 1997; Halanych et al., 1998). One explanation for this apparent discrepancy might be that tubes, such as those found in the Silurian fossil vent communities, did not belong to vestimentiferans, but rather to other tube-dwelling polychaetes. However, it is also possible that the choice of the molecular clock used to estimate the age of the vestimentiferan radiation from the molecular data, was simply not appropriate. Opportunities to accurately calibrate molecular clocks are relatively rare and cannot be generalized to all genes nor to all taxa.

Such an opportunity appeared during a phylogeographic study of the hydrothermal-vent ampharetid polychaete *Amphisamytha galapagensis* Zottoli, 1983, which is widely distributed in the Eastern Pacific (Chevaldonné et al., in prep.). Originally described from the Galapagos Rift, this worm was later found at most vent sites of the East Pacific Rise (EPR), but also on the northeastern Pacific ridge systems (Desbruyères, 1997). However, our ongoing molecular studies suggest that specimens from the northeastern ridges (Gorda, Juan de Fuca and Explorer ridges) should be assigned to a new cryptic sister-species

that is distinct from *A. galapagensis* but very closely related (Chevaldonné et al., in prep.). These related worms occupy distinct ridge systems that formerly were part of the Farallon-Pacific Ridge, which was disrupted by subduction under the North-American Plate about 28.5 MYA (Atwater, 1989; Severinghaus & Atwater, 1990). Thus, they provide a unique opportunity to investigate the processes that lead to allopatric speciation events between vent assemblages of both ridge systems (Tunnicliffe, 1988), and to calibrate the rate of molecular evolution in deep-sea annelids.

Material and methods

We chose to examine the mitochondrial gene coding for the Cytochrome Oxidase subunit I (mt*COI*) for the following reasons: (1) it is a single-copy gene in the mitochondrial genome; (2) it is a good marker for discriminating among congeneric species, at least for annelids; (3) there is a large sequence database for this particular gene in invertebrates; and (4) another marker of similar resolution, the mitochondrial 16S rRNA gene, could not be analysed for *Amphisamytha* using universal primers. The fragment (ca. 660 bp) of the *COI* gene used in this study was amplified using universal primers (see Black et al., 1997). Some of the sequences used here were retrieved from GenBank (Table 1).

We searched for pairs of sister-taxa from both sides of the California mainland (Gorda, Juan de Fuca and Explorer ridges to the north, Guaymas basin, EPR and Galapagos Rift to the south). In addition to the pair of cryptic *Amphisamytha* species mentioned above, two species of Alvinellidae, *Paralvinella grasslei* Desbruyères & Laubier,

Table 1. Pairs of hydrothermal vent species used to calibrate the *COI* clock.

Pairs of taxa	Origin of samples	Origin of DNA sequences
<i>Amphisamytha galapagensis</i> <i>Amphisamytha</i> n. sp. (JdF)	EPR / 13°N Juan de Fuca Ridge	Chevaldonné et al. in prep. Chevaldonné et al. in prep.
<i>Paralvinella grasslei</i> <i>Paralvinella palmiformis</i>	EPR / 9°50'N Juan de Fuca Ridge	Chevaldonné et al. in prep. GenBank #U74070
<i>Oasisia alvinae</i> <i>Ridgeia piscesae</i>	EPR / 21°N Gorda Ridge	GenBank #U74069 GenBank #U87979

1982, and *Paralvinella palmiformis* Desbruyères & Laubier, 1986, were identified as good candidates. They have long been considered sister-species based on their morphology (Desbruyères & Laubier, 1986) and genetics (Jollivet, 1996). One (*P. grasslei*) was sequenced, the other (*P. palmiformis*) was obtained from GenBank (Table 1). The Polynoidae are another polychaete family with potentially interesting sister-species, but the current taxonomic status of many of the described taxa needs to be revised before such pairs can be identified (S. Hourdez, pers. comm.). Finally, a pair of vestimentiferan worms was added as a comparison. The only species occurring on Northeast Pacific ridges, *Ridgeia piscesae* Jones, 1985 was retrieved from GenBank, as was *Oasisia alvinae* Jones, 1985, its closest relative from the EPR (Black et al., 1997) although this species is currently assigned to a different genus and family.

Pairwise comparisons of the nucleotide sequences were conducted, and Kimura 2-parameter (K2P) distances were estimated. Nucleotide sequences were translated into amino acid sequences and protein distances were also obtained (Dayhoff PAM matrix).

The mutation rate, or nucleotide substitution rate, can be estimated as $r = K / 2T$, where K is the K2P genetic distance, and T is the time of divergence, here multiplied by 2 to account for the age of each lineage (Li & Graur, 1991). Comparisons with previously published rate values require caution since authors have not always referred to mutation or nucleotide substitution but rather to pairwise sequence divergence per MY calculated as $r = K / T$. Here we restrict our use to the former definition.

Results and Discussion

Absolute pairwise differences in nucleotides and amino acids across the available alignments, as well as calculated distances, are reported in Table 2. There are significant differences, but K2P nucleotide distances seem to cluster around 10%. Protein distances are in good agreement for *Amphisamytha* and *Paralvinella* (about 1%) but, as previously reported (Black et al., 1997), there is a surprising identity between the amino acid sequences of the two vestimentiferans. This preliminary result will need to be confirmed by sequencing more individuals, but we can so far propose that *Oasisia alvinae* and *Ridgeia piscesae*, still currently placed in two different families, should be recognized as congeneric sister-species. Hurtado et al.

(2002, this volume) who have studied the molecular diversity within *Oasisia*, confirm that *Ridgeia* and *Oasisia* are sister lineages with very high bootstrap values.

The great interest in the pairs of species studied here is that we know there previously was a single contiguous ridge system in the Eastern Pacific, the Farallon-Pacific Ridge, which was interrupted ca. 28.5 MYA by its subduction under the North-American Plate (Atwater, 1989; Severinghaus & Atwater, 1990). The northern part of this

ridge system formed the Gorda, Juan de Fuca (JdF) and Explorer ridges, respectively, from south to north. The southern part is now known as the East Pacific Rise (EPR). This tectonic event caused a sudden isolation of northern and southern deep-sea hydrothermal vent communities living along the ridge axis. Although the subduction is relatively well dated, slight differences might have occurred in the timing of isolation between northern and southern populations, as a result of the different abilities of the taxa to disperse propagules, as well as their individual ecological requirements. However, gene flow was subsequently interrupted, leading to allopatric speciation, or vicariance, for virtually the entire vent assemblage (Tunnicliffe, 1988).

Substitution rates (r) can therefore be obtained using this time of divergence (T) and are reported in Table 2. Our estimates of substitution rate for the *COI* gene average ca. 0.2% per MY. Rates of ampharetids and vestimentiferans are very similar (0.23-0.25%). In alvinellids, we obtained a lower rate of 0.13% which seems to be primarily due to a higher proportion of synonymous changes in the nucleotide sequences of *Amphisamytha* and vestimentiferans compared to alvinellids. Also, alvinellid sequences represent the shortest alignments available to this study and may need to be reassessed in the future.

Table 2. *COI* mutation rates for pairs of hydrothermal annelid species. Nucl. div.: nucleotide divergence expressed as pairwise differences / total no. of nucleotides; K : genetic distance corrected according to the Kimura two-parameter model. AA diff.: pairwise difference in amino acid composition / total no. of amino acids. PAM: distance from protein sequences, based on Dayhoff PAM matrix. r : mutation rate (per MY).

Species pairs	Nucl. div.	(%) K	AA diff.	PAM (%)	r (%)
<i>A. galapagensis</i> <i>A. n. sp.</i> (JdF)	80/608	14.2	3/201	0.8	0.25
<i>P. grasslei</i> <i>P. palmiformis</i>	39/521	7.6	3/170	1.2	0.13
<i>O. alvinae</i> <i>R. piscesae</i>	76/631	13.1	0/210	0	0.23

The closure of the Panama seaway ca. 3-3.5 MYA (Knowlton & Weigt, 1998) has been used to calibrate

molecular clocks in several instances for marine species, and has also introduced some confusion into the evaluation of mt DNA evolutionary rates. Knowlton & Weigt (1998) argued that many of the published rate values based on transisthmian species pairs were overestimated, because of a failure to choose the most appropriate species pair, *i.e.* the last pairs to separate, such as mangrove species. The detailed study of trans-isthmian species of alpheid shrimps by Knowlton et al. (1993) provided a substitution rate estimate for the *COI* gene of 1.1-1.3% per MY (derived from pairwise divergence values of 2.2-2.3 % per MY), but Knowlton & Weigt (1998) later revised this estimate to 0.7% per MY based on a more appropriate mangrove species pair. Following their conclusions, we can consider this estimate as the best available to date for the crustacean *COI* gene, and that most (if not all) of the others are overestimated. Estimates of fish *COI* substitution rates based on a variety of transisthmian species are highly variable (Bermingham et al., 1997), but if we follow Knowlton & Weigt's (1998) recommendation, the safest rate estimate to be derived from the fish study is 0.25% per MY. This latter value is particularly close to our own estimates, and it is generally accepted that substitution rates in arthropods are usually high, which would account for the shrimp's *COI* rate being three times higher than for fish or annelids.

We presently lack data to evaluate possible peculiar characteristics of vent vs. non-vent taxa (e.g. higher selective constraint on protein-coding genes, codon bias, etc.), or any peculiarities of annelids as compared with other phyla, since this is the first molecular clock calibration proposed for this Phylum, to our knowledge. This clock calibration can, however, be useful for considering the divergence time between other hydrothermal polychaetes. For example, polynoid worms of the genus *Branchipolynoe* display genetic distances of ca. 17% (Chevaldonné et al., 1998; and in prep.) between the EPR species *B. symmytilida* and the Mid-Atlantic Ridge species *Branchipolynoe* n. sp. (MAR). In that case, dating of the closure of the Panama seaway is of little use considering that these are deep-sea species with a suspected benthic lecithotrophic development (Jollivet et al., 2000). Since the passage of deep waters between the Pacific and the Atlantic was interrupted long before the final closure 3-3.5 MYA, it is difficult to estimate a divergence time. Using the present calibration, this divergence appears to be very old (ca. 34 MYA), clearly predating the closure of the Isthmus of Panama. Other polynoid species isolated by the subduction of the Pacific-Farallon ridge, such as *Branchinotogluma* species, may provide confirmation when appropriate species pairs are found (S. Hourdez, pers. comm.). Finally, this new calibration, though still to be refined, allows us to look back into the possible age of divergence of the Vestimentifera. Considering a genetic distance < 27% (which seems to indicate no saturation) between perviate pogonophorans and Vestimentifera (Black et al., 1997), and applying a rate of 0.23% substitution per MY (Table 2), we find that the perviate/vestimentiferan split (ca. 59 MYA) indeed seems to have occurred quite recently. This estimate is remarkably similar to that of Halanych et al. (1998) based on ribosomal

gene sequences (ca. 50 MYA), and certainly less than the very conservative procedure (< 100 MYA) used by Black et al. (1997). Interestingly, the relatively young evolutionary ages recently inferred for several hydrothermal vent lineages may be in agreement with a scenario proposed by Jacobs & Lindberg (1998) where the global deep-water benthos, including vent faunas, was eliminated during anoxic/disoxic events at the Cenomanian/Turonian boundary and the latest Paleocene.

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