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Evidence for differences in the allozyme thermostability of deep-sea hydrothermal vent polychaetes (Alvinellidae): a possible selection by habitat

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ABSTRACT: Alvinellid polychaetes are, to date, restricted to deep-sea hydrothermal vents of the eastern and the western ridges of the Pacific Ocean. These organisms live in various sulfide-rich habitats, including the hottest part of the hydrothermal environment (i.e. chimneys). They experience transient anoxia, high levels of heavy metals and H_2S, natural radioactivity and temperatures ranging from 5 to 80°C which vary greatly with time. The Alvinellidae, as many vent organisms, have developed specific adaptations to cope with this harsh and unstable environment. Enzyme systems are good markers of the adaptation of ectotherms to temperature, which acts on both enzyme kinetics and protein denaturation. We estimated genetic distances between 11 alvinellid species using a data set of allozymes and studied *in vitro* allozyme thermostabilities of aspartate-amino transferase (AAT), glucose-6-phosphate isomerase (GPI) and phosphoglucomutase (PGM), which may play a role in orientating aerobic versus anaerobic metabolism pathways, for 8 species using the most common homozygous genotypes. Results show great genetic divergences between species living in distinct microhabitats as well as strong thermostability differences within and between species which also rely on different enzymatic strategies (phenotypic plasticity versus genetic variability). Allelic fitness to temperature in a highly fluctuating environment may explain the high level of polymorphism found in alvinellids and may have also provided sufficient genetic divergence between individuals living in distinct thermal regimes to produce speciation.

KEY WORDS: Deep-sea hydrothermal vents · Allozymes · Aspartate-amino transferase · Glucose-6-phosphate isomerase · Phosphoglucomutase · Temperature · Selection

INTRODUCTION

Fauna inhabiting deep-sea hydrothermal vents may experience high temperatures (Chevaldonné et al. 1991, 1992), natural radioactivity (Cherry et al. 1992), hypoxia and high concentrations of sulfide (Johnson et al. 1988), ammonia and heavy metals which greatly exceed those recorded for other marine or terrestrial environments (Edmond et al. 1982, Michard et al. 1984). These environmental conditions seem to be highly selective and able to induce peculiar adaptations (Felbeck 1981, Powell & Somero 1986, Arp et al. 1987, Dahlhoff & Somero 1991, Dixon et al. 1992). The environment is also characterized by great spatial and temporal instabilities due to both tectonic and magmatic events or the dynamic properties of the hydrothermal convection through the basaltic crust of the fast spreading centers (Hessler et al. 1988, Watremez & Kervevan 1990, Haymon et al. 1993, Jollivet 1993). The hydrothermal activity shifts along the spreading axes and generates numerous short-lived vents (1 to 100 yr: Lalou 1991), thus causing catastrophic and chaotic extinctions in populations which induce the continuous need to colonize new active vents (Hessler et al. 1988, Tunnicliffe & Juniper 1990, Jollivet 1993).

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The Alvinellidae is a family of polychaete worms restricted to the deep-sea hot hydrothermal vents located along the oceanic ridges of the eastern and western Pacific. A total of 12 species have so far been collected and described from vents of the East Pacific Rise including the Guaymas basin (5 species: Desbruyères & Laubier 1980, 1982, 1986, 1991), the ridges of the northeastern Pacific (4 species: Desbruyères & Laubier 1986, Detinova 1988, Tunnicliffe et al. 1993) and the ridges of the western Pacific back-arc basins (3 species: Desbruyères & Laubier 1989, 1993, Miura & Ohta 1991).

Alvinellid polychaetes are sedentary worms and live inside a tube or a mucus sheath. Most of them live on or at the base of the tubes of vestimentiferan worms in areas of diffuse low-temperature (5 to 25°C) venting, but a few others (*Alvinella pompejana*, *Alvinella caudata*, *Paralvinella sulfincola* and *Paralvinella hessleri*) live on the walls of hydrothermal chimneys where hot, anoxic, sulfide-rich fluid is mixing with cold (2°C) oxygenated seawater. These latter species inhabit the hottest zones of the hydrothermal-vent environment (Desbruyères et al. 1982, Chevaldonné & Jollivet 1993).

Recently, Tunnicliffe et al. (1993) and Jollivet et al. (1995) reported that these organisms display a genetic variability higher than that of most eucaryotes (Hartl 1988): they recorded polymorphism varying from 30 to 75% under the 5% criterion and observed heterozygosities from 0.1 to 0.3. They also reported a strong tendency towards a heterozygote deficiency systematically occurring at the same loci in nearly all the populations. Such a genetic structure does not fit with repeated founder effects which could be expected both from the vent patchiness and the low dispersal ability of alvinellids. Indeed, these polychaetes exhibit a peculiar reproductive behaviour and a development which may result in a short or absent planktonic phase (viz. larval dispersal) (Desbruyères & Laubier 1986, 1991, McHugh 1989, Zal et al. 1994, 1995). The heterozygote deficiencies might be better explained as an adaptive response of the polychaetes to the high temporal fluctuations of the vent conditions (Johnson et al. 1988, Little et al. 1988, Chevaldonné et al. 1991).

On the other hand, the genetic differentiation of alvinellid populations separated by distances varying from several metres to more than 1000 km is of the same magnitude for all the alvinellid species collected at vents of the East Pacific Rise and the ridges of the northern Pacific (Tunnicliffe et al. 1993, Jollivet et al. 1995). These results strongly support the hypothesis that genetic drift enforced by high rates of extinction/recolonisation is balanced by a uniform selective pressure stemming from the chemical and thermal homogeneity of vents on a large spatial scale (viz. East Pacific Rise; Edmond et al. 1982, Michard et al. 1984).

The aim of this study was to investigate the possible role of temperature both on the maintenance of a genetic structure and on the promotion of the genetic isolation of the alvinellid populations sampled from various microhabitats. To achieve this aim, the genetic relationships of 11 alvinellid species were analysed using a set of enzyme loci, and a comparative study was performed on the allozyme thermostability of the most common homozygous genotypes using 2 polymorphic enzyme loci, *Aat* (aspartate-amino transferase) and *Pgm* (phosphoglucomutase), and the monomorphic *Gpi* locus (glucose-6-phosphate isomerase) which is diagnostic in alvinellids.

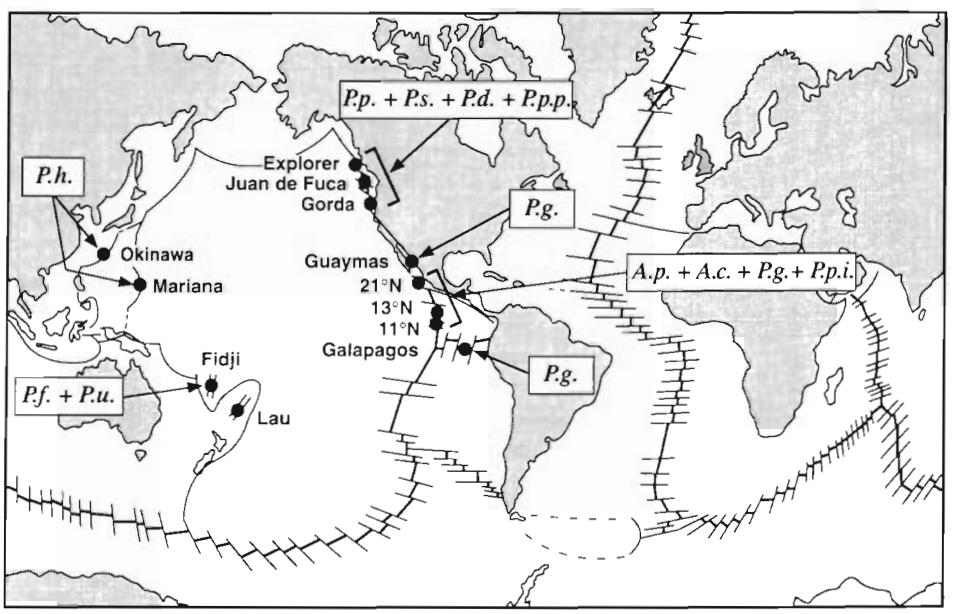

Fig. 1 Map of the deep-sea hydrothermal-vent areas where alvinellid species have been sampled. (*A.c.*) *Alvinella caudata*; (*A.p.*) *A. pompejana*; (*P.d.*) *Paralvinella dela*; (*P.f.*) *P. fijiensis*; (*P.g.*) *P. grasslei*; (*P.h.*) *P. hessleri*; (*P.p.*) *P. palmiformis*; (*P.p.i.*) *P. pandorae irlandei*; (*P.p.p.*) *P. p. pandorae*; (*P.s.*) *P. sulfincola*; (*P.u.*) *P. unidentata*

MATERIALS AND METHODS

Sample collection. Alvinellid samples were collected at deep-sea hydrothermal vents of the East Pacific Rise ('Hydronaut' 1987 and 'MMVT' 1990 cruises), the Juan de Fuca and Gorda ridge complex of the northern Pacific ('Axial Seamount Hydrothermal ExpeditionS, ASHES' 1986, 1987, and 'Gorda Ridge Expedition' 1988), and the western Pacific ridges of the Mariana and North Fiji back-arc basins ('Mariana Trench Vent Expedition, MTVE' 1987 and 'Starmer' 1989 cruise) (Fig. 1). The number of individuals used per species as well as location, depth and vent habitat are listed in Table 1. At least 3 main habitats have been distinguished: (1) the walls of active hydrothermal-vent chimneys, (2) the top of the tubes of vestimentiferan worms, and (3) basaltic cracks at diffuse-venting areas or at the base of active chimneys. Specimens were sampled using the hydraulic arms of the deep-sea manned submersibles Nautile and Alvin, brought to the surface in an insulated box, and frozen in liquid nitrogen.

Electrophoresis. Each piece of tissue was homogenized in an equal volume of a 0.01 M Tris/HCl pH 6.8 extraction buffer containing 2 mM EDTA, 0.5 mM NADP and 0.05% β-mercaptoethanol. Homogenates were centrifuged for 30 min at 25000 × *g* and the supernatant separated into 2 aliquots, one for the electrophoresis identification of allozymes and the other for biochemical experiments. EDTA was used in excess to chelate heavy metals found at high levels in the worms' tissues. Electrophoresis was conducted for 4 to 6 h at 80 mA using 11% starch gels refrigerated between 2 ice-water cooling plates using 2 continuous buffer systems: tris-citrate pH 8.0 and tris-HCl pH 8.5, and 2 discontinuous buffer systems: tris-citrate pH 6.7/6.3 and tris-citrate-borate pH 8.7/8.2. The *Alvinella pompejana* specimens collected at the site Pogonord in 1987 (see Table 1) were arbitrarily chosen as our reference population, and the following 14 enzyme loci were examined. Acid phosphatase (EC 3.1.3.2, locus *Acp*), aconitase (EC 4.2.1.3, loci *Acoh-1*, *Acoh-2*), aspartate-amino transferase (EC 2.6.1.1, locus *Aat*), adenylate kinase (EC 2.7.4.3, locus *Ak*), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, locus *Gapdh*), glucose-6-phosphate isomerase (EC 5.3.1.9, locus *Gpi*), isocitrate dehydrogenase (EC 1.1.1.42, locus *Idh-1*), cytosolic leucine aminopeptidase (EC 3.4.11.1, loci *Cap-1*, *Cap-2*), malate dehydrogenase (EC 1.1.1.37, locus *Mdh-1*), mannose-6-phosphate isomerase (EC 5.3.1.8, locus *Mpi*), phosphoglucomutase (EC 5.4.2.2, locus *Pgm*) and superoxide dismutase (EC 1.15.1.1, locus *Sod-1*) were scored for each individual. Alleles were numbered according to their anodal

Table 1. Location and habitat characteristics of 11 alvinellid species collected along the oceanic ridges of the Pacific. N: number of individuals sampled; *generic and specific name of a vestimentiferan tube-worm which provides a suitable habitat for an alvinellid species; EPR: East Pacific Rise

Species	Site	N	Location	Depth	Habitat
Alvinella pompejana	Pogonord (13°N/EPR)	20	12°48.61'N 103°56.62' W	2635 m	Top of the chimney
Alvinella caudata	Pogonord (13°N/EPR)	18	12°48.61'N 103°56.62'W	2635 m	Top of the chimney
Paralvinella grasslei	Genesis (13°N/EPR)	32	12°48.58'N 103°56.47'W	2635 m	Tubes of *Riftia pachyptila**
Paralvinella pandorae irlandei	Genesis (13°N/EPR)	35	12°48.58'N 103°56.47'W	2635 m	Cracks at the base of the tubes of *Tevnia jerichonana**
Paralvinella palmiformis	Nesca (Gorda Ridge)	48	41°00.40'N 127°29.30'W	3250 m	Tubes of *Ridgeia piscesae**
Paralvinella sulfincola	Lamphere (Juan de Fuca Ridge)	38	45°59.00'N 130°03.80'W	1575 m	Top of the chimney
Paralvinella pandorae pandorae	Max (Juan de Fuca Ridge)	35	45°55.50'N 130°02.50'W	1545 m	Cracks at the base of the tubes of *Ridgeia piscesae**
Paralvinella hessleri	Illium (Mariana Ridge)	42	18°12.80'N 144°42.40'E	3595 m	Base of the chimney
Paralvinella dela	Bouquet (Juan de Fuca Ridge)	12	45°55.50'N 130°02.50'W	1545 m	Cracks at the base of the tubes of *Ridgeia piscesae**
Paralvinella fijiensis	White Lady (North Fiji Basin)	6	16°59.50'S 173°55.47'W	2000 m	Top of the chimney
Paralvinella unidentata	White Lady (North Fiji Basin)	7	16°59.50'S 173°55.47'W	2000 m	Base of the chimney

mobility relative to the most frequent allele. The staining recipes were those provided in Harris & Hopkinson (1976) and Pasteur et al. (1987).

Thermostability theoretical background. Enzyme systems are good markers of the adaptation of ectotherms to temperature, which acts both on enzymatic rates and protein denaturation. Thermosensitivity of enzymes can be assessed by searching for the temperature for which enzyme activity is optimal, by following the evolution of the K_m (an indicator of the enzyme affinity for its substrate) with temperature (Hochachka & Somero 1984), or by following the protein stability with increasing incubation temperatures through changes in the residual enzyme activity (Fullbrook 1983). Enzymatic thermostability can be measured at constant pH and temperature by following the alteration in time of the enzyme. For our purposes, the time of incubation was set to 1 h (time for which all the allozymes displayed 20% of their original activity at 45°C) and incubations were performed at different temperatures.

Enzyme preparation. Thermostability was assessed in 8 alvinellid species for the most common homozygous genotypes encoded for aspartate-amino transferase (AAT: EC 2.6.1.1; 7 genotypes), glucose-6-phosphate isomerase (GPI: EC 5.3.1.9; 6 genotypes) and phosphoglucomutase (PGM: EC 5.4.2.2; 9 genotypes). These enzymes play a key role in glycolysis and anaerobic fermentative metabolism pathways, such as proteolysis and neoglucogenesis, and usually display allozymes known from shallow-water organisms to have a differential tolerance to temperature and pollutants (Hummel & Patarnello 1994). Aspartate-amino transferase (AAT) (or glutamate oxaloacetate transaminase, GOT) is a transaminase catalysing the reversible amino group transfer from L-aspartic acid and ketoglutaric acid to L-glutamate and oxaloacetate, L-glutamate often being an ammoniac carrier. Phosphoglucomutase (PGM) catalyses the interconversion of glucose-1-phosphate and glucose-6-phosphate in glycolysis and neoglucogenesis. Glucose-6-phosphate isomerase (GPI) catalyses the reversible isomerization of fructose-6-phosphate and glucose-6-phosphate in glycolysis and may provide a suitable substrate for neoglucogenesis. Kinetic differences between allozymes of these enzyme systems both *in vivo* and *in vitro* may provide adaptation to transient hot and hypoxic conditions through individual fitness differences. For *Alvinella* spp. which have epibiotic bacteria on their dorsal part (Desbruyères & Laubier 1986), and for species which have a large maximum size, enzyme assays were prepared only from the gills and anterior part of the body. At least 3 replicates corresponding to individuals having the same homozygous genotypes for at least 1 of the enzymes were selected for each species using starch-gel zymograms. For the small alvinellid species (i.e. *Paralvinella hessleri*), individuals were totally homogenized, and, in order to have a reasonable amount of crude enzyme extract per genotype, the aliquots containing the same homozygous genotype were grouped into 3 pools of 3 individuals. In addition, the protein concentration of each enzyme extract was measured according to the method of Lowry et al. (1951) to specifically express the enzyme activity (optical density: OD unit mg^{-1} protein).

Allozyme incubation and enzyme assays. Enzymatic mixtures were diluted in Tris-HCl 0.1 M pH 8.0 buffer (1:6) and sub-divided into 0.3 ml aliquots which were simultaneously incubated for 1 h at 4, 20, 35, 45, 50, 55, 60 and 65°C. Experiments were also conducted with fresh enzyme extracts at 4, 60, 62, 68 and 72°C when allozymes showed a strong resistance to temperature. The NAD appearance or the NADH disappearance rates were simultaneously measured at 340 nm using a 35°C-thermostated, multi-cuve spectrophotometer (UVIKON 930, Kontron) for each set of thermal treatments including 2 controls, one without substrate and one without enzyme extract. Assays were repeated using increasing amounts of extract (45, 90, 120 µl) to minimize errors associated with the calculation of the enzyme activity. The final reaction mixtures (2.5 ml) consisted of AAT [aspartic acid (215 mM), ketoglutaric acid (15 mM), pyridoxal-5-phosphate (0.6 mM), NADH (0.55 mM) and malate dehydrogenase (10 units ml^{-1})], GPI [fructose-6-phosphate (6.5 mM), $MgCl_2$ (20 mM), NAD (1 mM) and glucose-6-phosphate dehydrogenase (5 units ml^{-1})], and PGM [glucose-1-phosphate (40 mM), $MgCl_2$ (20 mM), NAD (1 mM) and glucose-6-phosphate dehydrogenase (5 units ml^{-1})], all dissolved in Tris-HCl 0.1 M pH 8.0 buffer. Concentrations of NAD or NADH, the other substrates and co-enzymes (G-6-PDH or MDH) were previously adjusted to be optimal. We calculated the percentage of the incubated-extract residual activity (RA%) for each allozyme using the formula $RA\% = 100(A_0 - A_T)/A_0$ in which A_T refers to the enzyme activity (OD unit mg^{-1} protein) measured spectrophotometrically at 35°C after a previous 1 h incubation at a selected temperature and A_0 to the initial enzyme activity.

RESULTS

Genetic distances

The allelic frequencies of each alvinellid sample are given in Jollivet (1993) and have been published in Tunnicliffe et al. (1993) and Jollivet et al. (1995) for *Alvinella pompejana*, *A. caudata*, *Paralvinella grasslei*, *P. palmiformis* and *P. sulfincola*. The unbiased Nei's

Table 2. *Alvinella* and *Paralvinella* spp. Unbiased Nei's genetic identities (above the diagonal) and distances (below the diagonal) estimated from alvinellid species pairwise comparisons using a set of 14 enzyme loci

Species	*A. pompejana*	*A. caudata*	*P. sulfincola*	*P. fijiensis*	*P. grasslei*	*P. palmiformis*	*P. p. pandorae*	*P. p. irlandei*	*P. unidentata*	*P. hessleri*	*P. dela*
A. pompejana	–	0.254	0.129	0.093	0.014	0.014	0.015	0.010	0.022	0.062	0.086
A. caudata	1.376	–	0.106	0.053	0.082	0.126	0.074	0.016	0.012	0.022	0.310
P. sulfincola	2.049	2.248	–	0.400	0.095	0.075	0.071	0.098	0.160	0.179	0.088
P. fijiensis	2.391	2.953	0.918	–	0.170	0.134	0.014	0.048	0.143	0.123	0.089
P. grasslei	4.676	2.523	2.382	1.782	–	0.419	0.100	0.155	0.115	0.107	0.053
P. palmiformis	4.865	2.077	2.649	2.015	0.872	–	0.101	0.082	0.092	0.139	0.070
P. p. pandorae	4.215	2.598	2.870	4.283	2.307	2.295	–	0.545	0.362	0.313	0.328
P. p. irlandei	4.628	4.168	2.006	3.036	1.866	2.504	0.606	–	0.433	0.281	0.243
P. unidentata	4.476	4.487	1.837	1.947	2.172	2.388	1.015	0.836	–	0.426	0.232
P. hessleri	2.788	3.843	1.722	2.097	2.240	1.977	1.161	1.270	0.854	–	0.482
P. dela	2.449	1.168	2.441	2.418	2.944	2.668	1.116	1.415	1.462	0.731	–

genetic identities and distances (Nei 1978), calculated using the program BIOSYS-1 (Swofford & Selander 1989), are given in Table 2 and provided the data base for an UPGMA phenogram (Fig. 2). Genetic distances obtained between species varied from 0.61 (subspecific level) to 4.87 (generic level). The 2 genera are monophyletic and the *Paralvinella* genus is cleaved into 3 sister groups. Each group contains a set of allopatric species which are morphologically alike and live in similar microhabitats. We estimated the divergence time between the 3 sister groups of *Paralvinella* using Nei's formula $I_{XY} = I_{0XY} \cdot e^{-2\alpha t}$ where I_{XY} and I_{0XY} correspond to current and initial genetic identities of the populations X and Y, α is the mutation rate and t the divergence time. The calculation was done assuming that both separation between the subspecies *P. pandorae pandorae* and *P. p. irlandei* and the sibling species *P. palmiformis* and *P. grasslei* occurred 35 million years ago when the Farallon plate was subducted below the American plate, resulting in the separation of the northern and the southern East Pacific ridges (Tunnicliffe 1988). The correlation between genetic distance and divergence time indicates that the cleavage of the 3 sister groups of *Paralvinella* occurred before the opening of the Atlantic ocean, during the Mesozoic, 150 million years ago.

Thermostability of allozymes

Figs. 3, 4 & 5 display the thermostability curves obtained using allozymes of AAT, GPI and PGM for 8

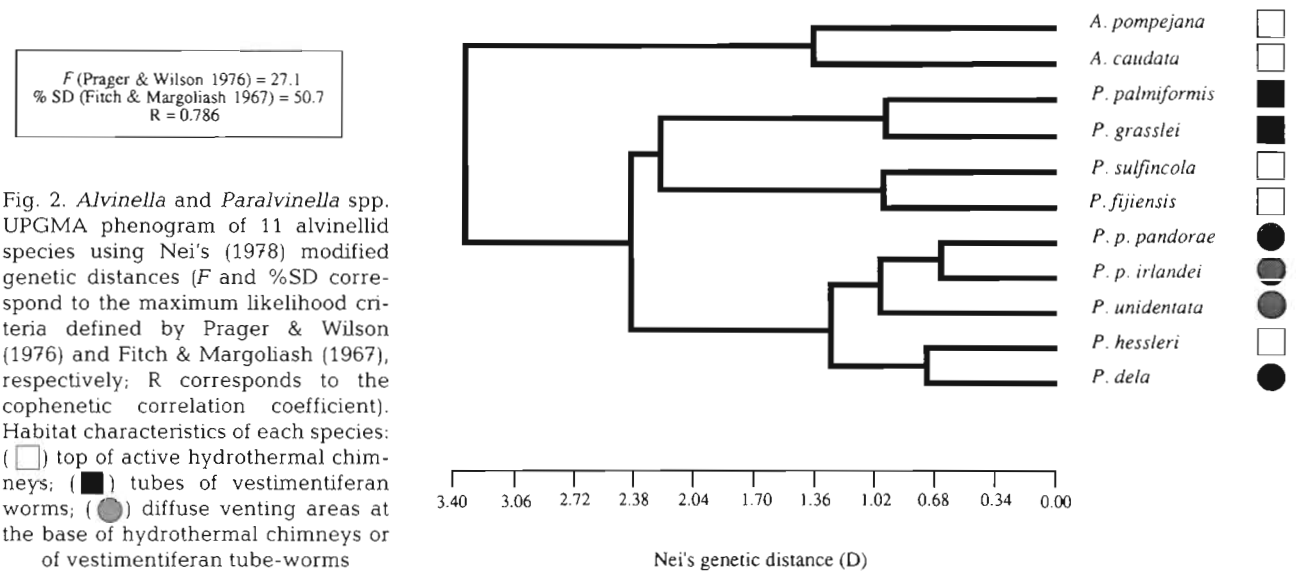

Fig. 2. *Alvinella* and *Paralvinella* spp. UPGMA phenogram of 11 alvinellid species using Nei's (1978) modified genetic distances (*F* and %SD correspond to the maximum likelihood criteria defined by Prager & Wilson (1976) and Fitch & Margoliash (1967), respectively; R corresponds to the cophenetic correlation coefficient). Habitat characteristics of each species: (□) top of active hydrothermal chimneys; (■) tubes of vestimentiferan worms; (●) diffuse venting areas at the base of hydrothermal chimneys or of vestimentiferan tube-worms

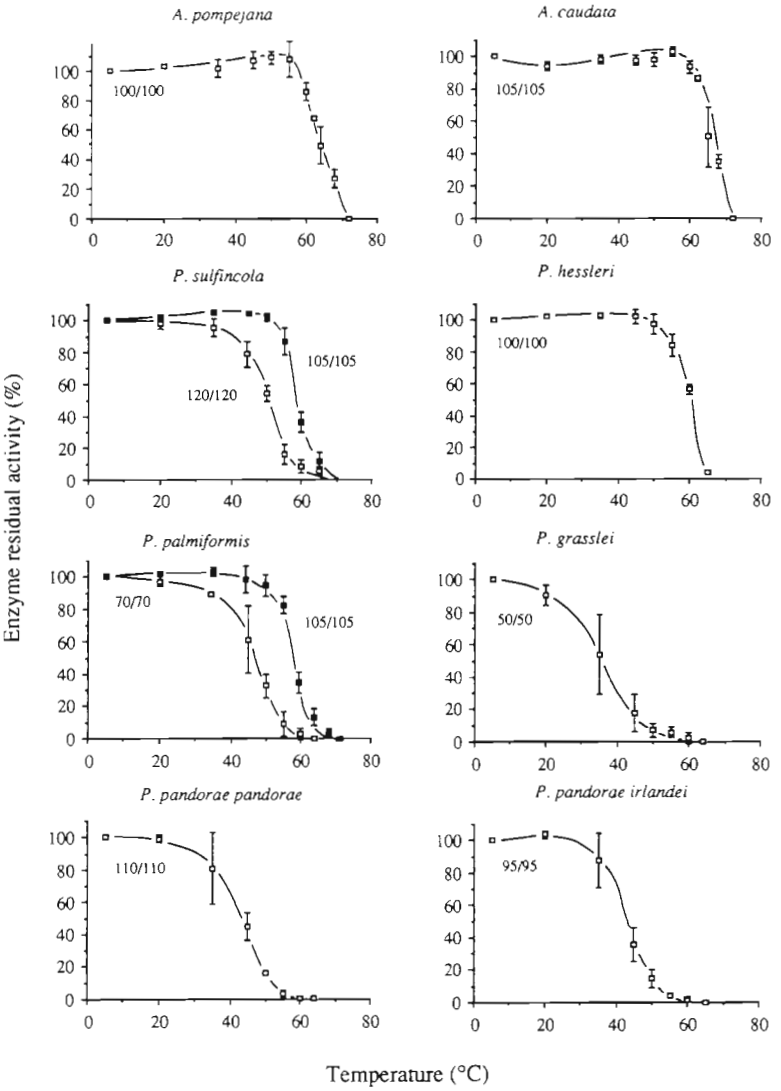

Fig. 3. *Alvinella* and *Paralvinella* spp. Thermostability curves obtained for allozymes corresponding to homozygous genotypes at the locus *Aat* in 8 alvinellid species (bars correspond to the standard errors associated with the percentage of residual activity)

alvinellid species. The shape of the curve varies slightly according to the enzyme system. Allozymes of AAT and GPI are characterized by a plateau followed by a rapid decrease of the enzyme activity with increasing incubation temperatures whereas the curves obtained for the allozymes of PGM are sigmoidal. Furthermore, the half-life time ($t_{1/2}$) of AAT allozymes incubated at 45°C was respectively twice and 4 times greater than those of GPI and PGM when we attempted to fix the incubation time. To compare the thermostability of allozymes encoded by distinct genotypes within and between alvinellid species, we used $T_{80\%}$ and $T_{20\%}$, temperatures at which the enzyme conserves 80% and 20% of its initial activity after 1 h incubation. $T_{80\%}$ usually sets the limit at which the enzyme begins to denature and $T_{20\%}$ sets the limit at which the enzyme is totally denatured. Results are summarized in Table 3 and Fig. 6 in which $T_{80\%}$ is plotted against $T_{20\%}$ to distinguish species in terms of 'hot' versus 'cold' adapted genotypes.

Intraspecific variation. Monomorphic species at either *Aat*, *Gpi* or *Pgm* loci always exhibit an allozyme which displays a $T_{80\%}$ similar to that of the highest thermoresistant allozymes of their polymorphic counterparts. This is especially the case for most GPI allozymes and the AAT allozymes of the 2 *Alvinella* species studied here. In the other cases, species are usually polymorphic but alternative alleles are often rare and found only as heterozygotes. As a consequence, it was not possible to assess the thermostability of the homozygous genotypes. However, in a few cases, alternative alleles were more frequent within samples and allowed us to find enough homozygous genotypes to make comparisons (see Fig. 3: $Aat^{70/70}$, $Aat^{105/105}$ and $Aat^{120/120}$ in *Paralvinella palmiformis* and *P. sulfincola*; and Fig. 5: $Pgm^{66/66}$, $Pgm^{78/78}$, $Pgm^{90/90}$ and $Pgm^{100/100}$ in *P. sulfincola* and *A. pompejana*). Results show the occurrence of at least 2 allozymes displaying $T_{80\%}$ differences ranging from 10 to 20°C, which indicates that allozyme thermostability varies greatly within species from individual to individual according to genotype.

Interspecific variation. Enzyme activities of the 2 *Alvinella* species studied here still persist after 1 h incubation at temperatures exceeding 50°C (GPI) and 60°C (AAT). *Paralvinella hessleri* and *P. sulfincola* also exhibit a $T_{80\%}$ greater than 45°C (GPI) and 55°C (AAT), whereas other congeneric species have much lower values (Table 3). This is particularly obvious in Fig. 6 in which all the species living on the walls of active hydrothermal chimneys (*A. pompejana*, *A. caudata*, *P. sulfincola* and *P. hessleri*) display thermoresistant allozymes at least for the AAT and GPI enzyme systems, whereas species living in 'cooler' diffuse venting conditions (*P. grasslei*, *P. palmiformis*, *P. pandorae pandorae* and *P. p. irlandei*) are characterized by somewhat more sensitive alleles. The PGM allozymes appear to be less informative and do not provide reliable markers to distinguish ecotypes because most of the species are polymorphic

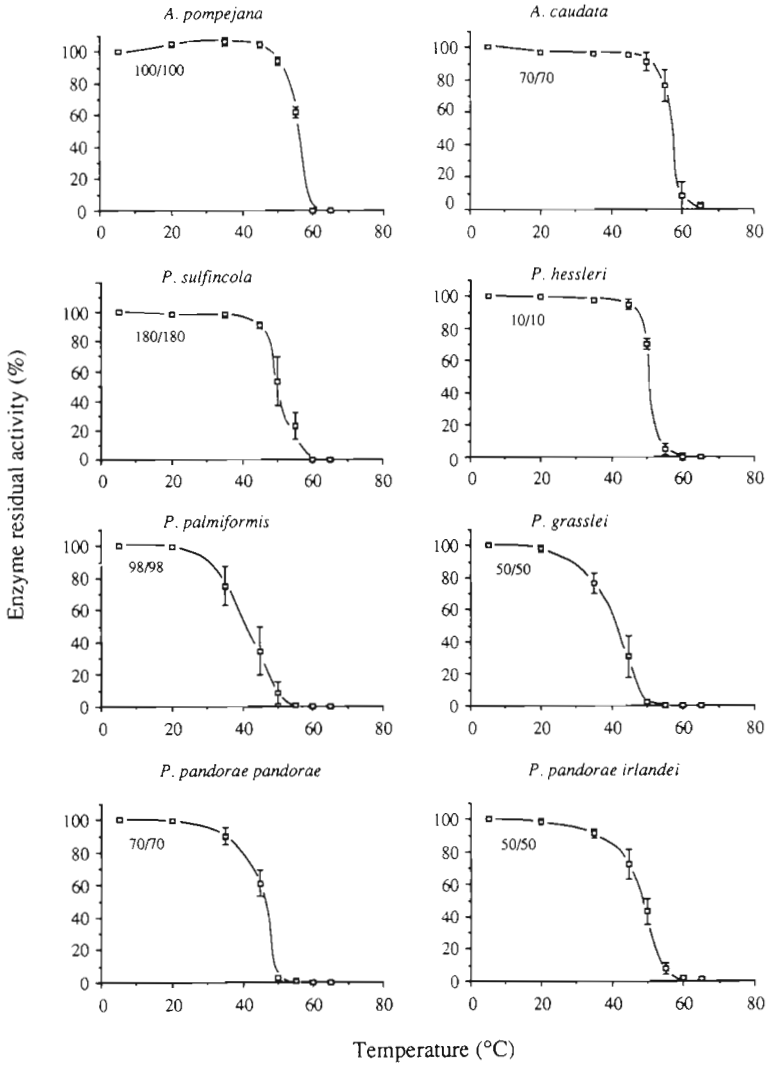

Fig. 4. *Alvinella* and *Paralvinella* spp. Thermostability curves obtained for allozymes corresponding to homozygous genotypes at the locus *Gpi* in 8 alvinellid species (bars correspond to the standard errors associated with the percentage of residual activity)

and exhibit 2 or 3 equifrequent alleles coding for strong differences in the protein thermostability.

DISCUSSION

Genetic variability of alvinellids and short- and long-term fluctuations in venting

Protein polymorphism may indicate a genetic response of ectotherm populations to selection in various environments. Ectotherms, especially marine invertebrates, need to regulate their body temperature in order to improve their metabolic functions, especially enzyme kinetics. Physiological adaptations of organisms to changing environments can be achieved by a phenotypic regulation of the genetic expression or by reduction of the genetic diversity of populations through elimination of individuals lacking the advantageous alleles (Hochachka & Somero 1984). As a consequence, the adaptive nature of enzyme genotypes to temperature has been largely supposed to explain deviations from the Hardy-Weinberg equilibrium in shallow-water populations subjected to ecological or anthropological stresses (Nevo et al. 1977, Patarnello et al. 1989, 1991).

At deep-sea vents, temperature is a semiconservative factor of dilution between the acidic, hot, hydrothermal fluid and the cold, oxygenated seawater. At a local scale, it is well correlated with H_2S concentrations and negatively correlated with oxygen concentrations (Johnson et al. 1988) and thus reflects changes for most environmental parameters affecting the physiology of the vent organisms. Our results demonstrate that some alleles may encode for differences in the allozyme sensitivity to temperature within alvinellid species exhibiting polymorphism. Such results have already been reported by Place & Powers (1979), who found that the cytosolic allozymes LDH^{BaBa} and LDH^{BbBb} of the teleost fish *Fundulus heteroclitus* are sensitive to temperature, and by Wilkins et al. (1978), who found thermoresistant and thermosensitive allozymes in shallow-water limpets and littorinid gastropods (which might explain their genetic diversity). Rapid changes in temperature, exposure to waves and salinity with time are therefore known to be responsible for increasing genetic variability in marine invertebrates living in the intertidal zone or in estuaries (Hummel & Patarnello 1994). Similarly, such an allelic fitness may explain the high levels of genetic variability recorded in alvinellids (Tunnicliffe et al. 1993, Jollivet et al. 1995).

The alvinellid environment is not only characterized in terms of 'hot' and 'warm' habitats but also in changes with time. Time series of temperature recorded at the surface of alvinellid colonies indicate 2 kinds of temporal fluctuations wherein a high-frequency signal is superimposed on one with a much longer period clearly associated with tidal cycles (Chevaldonné et al. 1991). Temperature increases dramatically 5 to 10 cm inside a colony. Desbruyères et al.

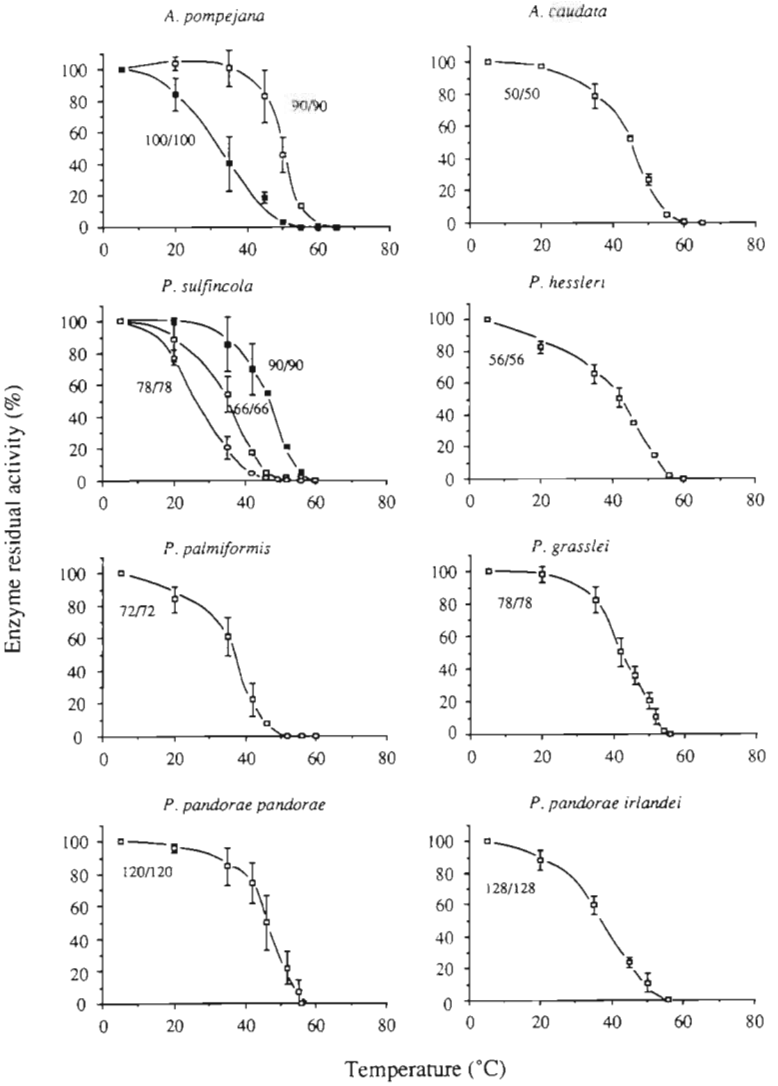

Fig. 5. *Alvinella* and *Paralvinella* spp. Thermostability curves obtained for allozymes corresponding to homozygous genotypes at the locus *Pgm* in 8 alvinellid species (bars correspond to the standard errors associated with the percentage of residual activity)

(1982) reported a temperature range from 20°C (at the tube openings) to 100°C (10 cm inside). More recently, Chevaldonné et al. (1992) provided *in situ* discrete temperature measurements simultaneously recorded on videotapes, which varied from 20 to 45°C when the probe was placed close to the gills of the worms and from 40 to 80°C when the probe was inserted 2 to 3 cm inside a tube. They also reported a temperature measurement at 105°C while a dislodged, live *Alvinella pompejana* was coiled around the probe for a few minutes without showing any change in its behaviour. Thus, it is likely that short-term fluctuations in the oxygen concentration and temperature could subsequently affect one of the 2 homozygous genotypes at a non-neutral diallelic locus, giving a selective advantage to the other one and vice versa. As a consequence, heterozygotes will get a selective advantage because of their less energy-demanding maintenance, as predicted by Koehn & Bayne (1989), when environmental conditions vary greatly.

In addition, environmental conditions also evolve rapidly during the lifetime of a vent: *in situ* observations have shown that a vent may evolve from birth to death within a decade (Jollivet 1993). During that period, hydrothermal activity progressively decreases and the venting fluid becomes cooler and cooler until venting stops completely. As a consequence, the first colonizers may experience higher temperatures than other successive cohorts, and thus modifications in the frequency of thermosensitive alleles could arise with time. Such variations have been reported by Monti et al. (1986), who observed rapid changes in the malate dehydrogenase (*Mdh*) genotypic frequencies within a population of the shallow-water bivalve *Ruditapes decussatus* in response to increasing temperatures and hypoxia during the summer period. Similarly, the differential sensitivity to temperature of allozymes encoded by Aat^{105} and Aat^{120} seems to explain genotypic modifications observed by Tunnicliffe et al. (1993) in a population of *Paralvinella palmiformis* sampled before and after (1 yr) the vent site Mushroom unexpectedly warmed up. In that particular case, the frequency of the allele Aat^{105} increased from 0.042 to 0.433 within the same population of *P. palmiformis* between 1986 and 1981.

Microhabitats and alvinellid speciation

Genetic distances and their derived phenogram indicate important evolutionary divergences, which are consistent with relationships obtained using morphological characters (Desbruyères & Laubier 1991, 1993), among alvinellid species sampled so far. Even if allozymes are not the most appropriate indicators of the phylogenetic relationships between genera or highest taxa, such high genetic distances may stem from very old cleavages spurred by the oceanic plate movement. Indeed, some vent fauna is known to have direct ancestors from the early Mesozoic (Newman 1985), and fossil burials attributed to alvinellid polychaetes have been reported from sulfide ore structures dated

Table 3. *Alvinella* and *Paralvinella* spp. $T_{80\%}$ and $T_{20\%}$ (temperatures at which the enzyme conserves 80% and 20% of its initial activity) obtained for allozymes of the AAT, GPI and PGM for 8 alvinellid species. Temperatures were graphically estimated on the thermostability curves

Species	Aspartate-amino transferase			Glucose-6-phosphate isomerase			Phosphoglucomutase		
	Genotype	$T_{80\%}$	$T_{20\%}$	Genotype	$T_{80\%}$	$T_{20\%}$	Genotype	$T_{80\%}$	$T_{20\%}$
A. pompejana	*100/100*	61	68	*100/100*	52	58	*100/100*	23	44
							90/90	46	54
A. caudata	*105/105*	62	69	*70/70*	54	58	*50/50*	34	52
P. hessleri	*100/100*	56	63	*10/10*	48	54	*56/56*	24	52
P. sulfincola	*120/120*	46	54	*180/180*	48	56	*90/90*	36	54
	105/105	56	62				*78/78*	22	36
							66/66	24	42
P. pandorae irlandei	*95/95*	37	48	*50/50*	42	52	*120/120*	38	52
P. pandorae pandorae	*110/110*	36	49	*70/70*	40	48	*128/128*	28	44
P. grasslei	*50/50*	26	44	*50/50*	33	47	*78/78*	32	50
P. palmiformis	*70/70*	39	52	*98/98*	32	48	*72/72*	26	44
	105/105	53	60						
Mean		47.2	56.9		43.6	52.6		30.3	47.6
SE		(±3.8)	(±2.8)		(±2.9)	(±1.6)		(±2.3)	(±1.8)

from the Cretaceous and the Devonian periods (Haymon et al. 1984, Oudin & Constantinou 1984, Kuznetsov et al. 1991).

However, cinepatry (i.e. species isolation due to the oceanic plate movement) does not appear to be the only evolutionary process acting on hydrothermal-vent species isolation. Our calculations indicate that species which compose the genus *Paralvinella* evolved in 3 sister groups approximately when the 2 alvinellid genera diverged some 150 million years ago; such a divergence may have been favoured by different ecological constraints. Indeed, morphologically closely related species such as *P. palmiformis* and *P. sulfincola*, which have very different ecological preferenda, display a high level of genetic divergence (Tunnicliffe et al. 1993). In an opposite fashion, closely related species such as *P. dela* and *P. hessleri* or *P. sulfincola* and *P. fijiensis*, which live in similar habitats but have been geographically isolated for a long period of time (eastern Pacific versus western Pacific), display great genetic similarities. Desbruyères & Laubier (1993) have already proposed that the occurrence of morphologically closely related species of *Paralvinella* living in similar habitats over the 3 main hydrothermically active areas of the Pacific (eastern Pacific, northeastern Pacific and western Pacific) may be due to the

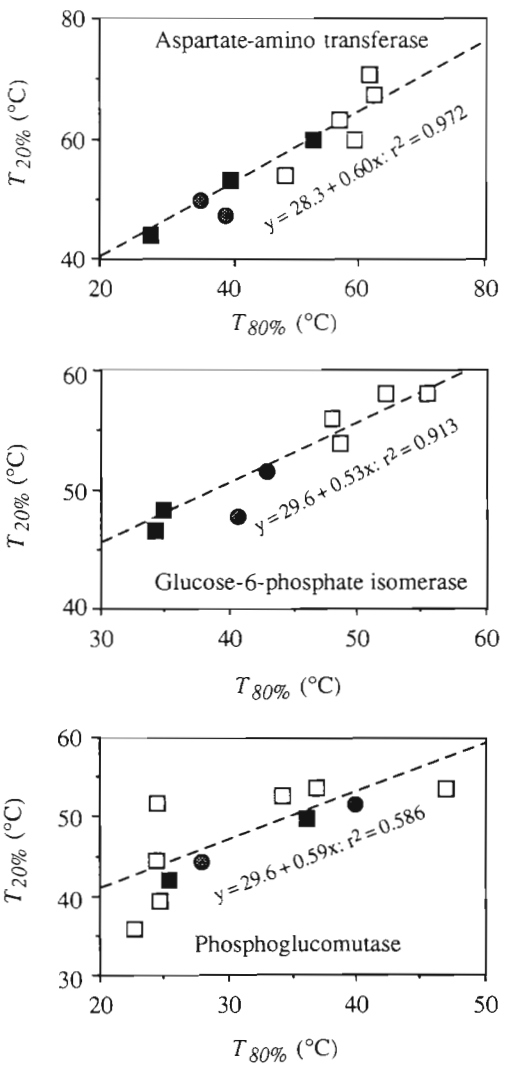

Fig. 6. Thermostability of AAT, GPI and PGM allozymes along a gradient of incubation temperatures obtained from a linear regression (r^2: correlation coefficient) against the temperatures $T_{20\%}$, at which an allozyme conserves 20% of its initial activity, and $T_{80\%}$, at which an allozyme conserves 80% of its initial activity. Symbols as in Fig. 2

parallel evolution of 3 ancestral types living in 3 distinct hydrothermal-vent habitats. As a consequence, we may infer that ecological constraints could have provided evolutionary forces which drove alvinellid species toward isolation.

Adaptive responses of allozymes to temperature have been previously proposed as explanations of speciation in 4 species of eastern Pacific barracudas living in different thermal environments (Graves & Somero 1982). To determine if functionally significant molecular evolution has occurred in response to different thermal microregimes, allozyme thermostabilities of the diagnostic locus (*Gpi*) and polymorphic loci displaying shared alleles between species (*Aat* and *Pgm*) were measured.

The level of thermal stability obtained from our set of enzymes appeared to be well correlated to the maximum temperature each alvinellid species might experience within its habitat. $T_{80\%}$ values, which ranged from 22°C ($Pgm^{78/78}$) to 62°C ($Aat^{105/105}$), are also in good agreement with several *in vitro* comparative physiological studies conducted on the body-temperature range that vent organisms could sustain. Haemoglobin O_2 affinity (Toulmond et al. 1990) and collagen melting-temperature (Gaill et al. 1991) studies conducted on the 2 *Alvinella* species gave an optimal temperature range of 20 to 50°C whereas results obtained from enzyme kinetics varied from 25 to 40°C (Dahlhoff & Somero 1991, Dahlhoff et al. 1991). However, caution must be taken when an attempt is made to interpret our thermostability values because (1) $T_{80\%}$ neither reflects the maximal nor the optimal temperature of enzymes and (2) we used crude instead of purified enzyme extracts which did not allow us to discard the influence of the cell micro-environment (i.e. the more or less important efficiency of proteases during incubation and/or the measurement of activity, presence or absence of specific inhibitors such as cytosolic heavy metals). Nevertheless, enzyme activities are reproducible across replicates and different enough between genotypes to discount the influence of the cell environment.

This study is therefore the first attempt to compare congeneric hydrothermal vent species living in 'hot' and 'warm' habitats. Results obtained for the AAT, GPI and PGM enzyme systems indicate that species living on the walls of hydrothermal vent chimneys display thermoresistant allozymes whereas species living in 'cooler' conditions (diffuse venting areas) display allozymes more sensitive to temperature. Moreover, there also exist electromorphic and thermostability convergences at the *Aat* and *Pgm* loci between alvinellid species which are not closely related but which live in similar microhabitats. Indeed, *Alvinella pompejana* and *Paralvinella sulfincola*, which both live on the walls of hydrothermal chimneys, share the allele Pgm^{90} which encodes for the most thermostable PGM allozyme ever recorded for alvinellid polychaetes.

Such results are in agreement with ecological and physiological data previously recorded on vent organisms, especially on alvinellid polychaetes. Dahlhoff & Somero (1991) have already predicted that deep-sea hydrothermal vent organisms living on chimneys emitting high temperature fluids have sustained body temperatures which are 20 to 25°C higher than those of vent species living in cooler diffuse vent habitats. Similarly, Dixon et al. (1992) found differences in the thermal stability of ribosomal DNA sequences of hydrothermal-vent organisms including several alvinellid species which fit our data very well: melting rDNA temperatures varying from 88°C (both species of *Alvinella*) to 82°C (*Paralvinella grasslei* and *P. pandorae*). Finally, Taghon (1988) reported great differences in the levels of polyunsaturated fatty acids between *P. palmiformis* and *P. sulfincola*, which was explained as a specific adaptive response of the latter species to increasing habitat temperature. As a consequence, although temperature strongly varies with time on short- and long-term scales, comparisons between congeneric paralvinellid species using enzyme thermostability indicate that differences in habitat are sufficient to favour molecular adaptations in functional genes which may result in species isolation.

In conclusion, we can infer from these results that temperature fluctuations and the aerobic/anaerobic alternation of hydrothermal vent conditions seem to play a determinant role in the evolution of the genetic structure of vent organisms. Vent instability may be responsible for maintaining a high genetic diversity of the alvinellids, whereas the spatial heterogeneity of the thermal regime within a vent site seems to have provided an evolutionary driving force to produce genetic divergences between species living in distinct microhabitats.

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