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I. Kappas · T. J. Abatzopoulos · N. Van Hoa
P. Sorgeloos · J. A. Beardmore

Genetic and reproductive differentiation of *Artemia franciscana* in a new environment

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Abstract The nature and speed of intraspecific genetic differentiation between the *Artemia* population native to San Francisco Bay (SFB) and populations resulting from introduction of SFB material into Vietnam (Vinh Chau, VC) have been investigated from a multidisciplinary perspective using reproductive characters, allozymes and mitochondrial DNA. Rapid divergence between SFB and VC *Artemia* is evident from an analysis of five reproductive traits at the temperatures of 26°C and 30°C. The VC strain seems to be better adapted to high temperature, as gauged by the significantly higher reproductive output displayed compared with SFB *Artemia*. Analysis of variance confirms the existence of a strong genetic component involved in the determination of encystment. Allozyme analysis (20 loci) of the same strains reveals considerable genetic differences between samples, cultured at the above temperatures. Combined probability values for tests of allele frequency homogeneity pooled over all polymorphic loci, at 30°C as well as between temperatures, are 0.015 and 0.026, respectively. In addition, cluster analysis shows evidence of differentiation of Vietnamese *Artemia* from the wild (SFB) stock as early as within a year after inoculation. Mitochondrial DNA RFLP markers show similar patterns of genetic differentiation to those seen with allozymes and reproductive traits. However, unlike allozymes, there is strong evidence of a reduction in haplotype diversity from 40.6% in SFB *Artemia* to 10.5% in the established VC strain. Although genetic drift could account for much of the depletion in gene diversity, firm indications are obtained of selective forces shaping the gene pool in the tropical conditions of Vietnam. The brine shrimp is an invaluable genetic system for fine-scale studies of microevolutionary divergence, and multidisciplinary studies are likely to be of both fundamental and practical value for such inquiries.

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I. Kappas (✉) · T. J. Abatzopoulos
Department of Genetics, Development and Molecular Biology,
Aristotle University of Thessaloniki,
541 24 Thessaloniki, Greece
E-mail: kappasi@hol.gr
Tel.: +30-2310-998301
Fax: +30-2310-998256

N. Van Hoa
Institute of Marine Aquaculture,
University of Can Tho, Can Tho, Vietnam

P. Sorgeloos
Laboratory of Aquaculture and Artemia Reference Center,
Ghent University, Rozier 44, 9000 Ghent, Belgium

J. A. Beardmore
School of Biological Sciences,
University of Wales Swansea, Singleton Park,
Swansea, Wales, SA2 8PP, UK

Introduction

Ecological genetics and reproductive biology have always been abutting subjects. However, their evolutionary purviews may be better appreciated through research on suitable organisms constituting model systems in which experimental ease, tractability, appropriate genetics and distinctive life cycle are combined.

Artemia is an anostracan crustacean, occurring on every continent except Antarctica (Triantaphyllidis et al. 1998). It is a typical inhabitant of inland salt lakes, coastal lagoons and solar saltworks (Persoone and Sorgeloos 1980), and the prime abiotic factor determining its presence is high salinity. Although *Artemia* is restricted to hypersaline biotopes, other factors such as temperature, ionic composition and biotic interactions also play an important role in the patterns of its distribution (see Van Stappen 2002). Within these biological communities there is considerable diversification with respect to permanence, seasonality and predictability of the environment (Lenz 1987). The brine shrimp is able to overcome the severe physiological demands imposed by these habitats, due to a set of various adaptations, the most salient of which is probably an interchangeable (diapausing cysts versus nauplii) life cycle.

Among the bisexual species (see Abatzopoulos et al. 2002a) of the *Artemia* genus, *A. franciscana* has been the most extensively studied. *A. franciscana* is endemic to the New World; however, both permanent and temporal populations also exist worldwide, due to introductions of different strains (Lenz and Browne 1991). Over the last 20 years or so research on natural populations of *A. franciscana* from most parts of its distribution as well as from transplanted populations has produced an abundance of data bearing on a variety of disciplines (see Abatzopoulos et al. 2002b, and references therein). Generally speaking, at a broad range of salinities, *A. franciscana* seems to be a better performer, in terms of survival and reproductive characteristics, than other bisexual and parthenogenetic strains (Wear and Haslett 1986; Wear et al. 1986; Triantaphyllidis et al. 1995). Regarding temperature, most strains do not survive <6°C or >35°C, and tolerance to different temperatures is strain specific (Browne et al. 1988). Temperatures in the mid-twenties are likely to be optimal for survival, and *A. franciscana* is a good performer in both low (Wear and Haslett 1986; Wear et al. 1986; Gajardo and Beardmore 1993) and high (Vos and Tunsutapanit 1979; Quynh and Lam 1987) temperatures. Triantaphyllidis et al. (1994) have shown that encysted embryos of *A. franciscana* retain their hatchability at 36–37°C, contrary to other species. Survival and reproduction are also heavily dependent on the interaction between salinity and temperature. Browne and Wanigasekera (2000) have rightly stressed the importance of this interaction by estimating a number of survival and reproductive traits in five species of *Artemia*, in an extended array of nine temperature–salinity combinations. Their results have shown that *A. franciscana* is both euryhaline and eurythermal, reproducing in most combinations of conditions. All the previous studies converge to the conclusion that *A. franciscana* exhibits high levels of phenotypic plasticity, thus enabling the attainment of adaptations to a wide range of ecological settings.

The literature on *Artemia* is also replete with molecular data on the intra- and interspecific diversity of the genus. Research in these fields was spurred by the same realisations of the importance in aquaculture, the extent of physiological variability and the distinctiveness of the biology of the brine shrimp. Until almost the mid-1990s most genetic investigations utilised allozymic analyses to address various issues, such as levels of polymorphism, population structure, patterns of geographic divergence and others (for reviews see Abreu-Grobois and Beardmore 1980, 1982; Abreu-Grobois 1987; Browne and Bowen 1991). Compared with other *Artemia* species, levels of heterozygosity for *A. franciscana* populations vary more widely (0.02–0.17, Abreu-Grobois 1983; Pilla 1992; Gajardo et al. 1995; Thomas 1995), while the mean genetic distance separating populations is on the order of 0.126 (Abreu-Grobois 1983). Similarly, levels of population substructuring for *A. franciscana* are higher (mean $F_{\mathrm{ST}}=0.24$, Beardmore and Abreu-Grobois 1983) than in other species. In a study of South American *A. franciscana* populations, Gajardo et al. (1995) reported a mean F_{ST} value of 0.38. All these data are a reflection of the levels of divergence and local differentiation between populations of the *A. franciscana* group. Over the last decade, interpretations of patterns of genetic differentiation and the distribution of diversity in several organisms have been considerably refined by the introduction of new molecular tools (see Avise 1994). The use of mitochondrial DNA (mtDNA) markers (Perez et al. 1994; Valverde et al. 1994; Thomas 1995) as well as nuclear sequences (Badaracco et al. 1995; Triantaphyllidis et al. 1997; Sun et al. 1999a, 1999b; Abatzopoulos et al. 2002a) have provided additional data, albeit mainly on the evolutionary relationships and levels of divergence between species of the genus. Nevertheless, Badaracco et al. (1995) demonstrated extensive polymorphism within populations of *A. franciscana* by principal coordinate analysis of randomly amplified polymorphic DNA (RAPD) markers. Also, Gajardo et al. (2004) utilised restriction analysis of the 16S rRNA mitochondrial gene and found significant F_{ST} values (91%) between Chilean *A. franciscana* populations.

It would not be an overstatement to say that the majority of distinctive features found in the genus (see Abatzopoulos et al. 2002b) are harboured within *A. franciscana* alone. This fact, combined with the presence of many natural or introduced populations around the world, makes *A. franciscana* an invaluable genetic system for fine-scale studies of microevolutionary divergence. On the basis of these kinds of inquiries, multidisciplinary approaches are particularly germane. Although their power has been demonstrated at the interspecific level (see Triantaphyllidis et al. 1997; Abatzopoulos et al. 2002a), the literature is relatively devoid of such surveys within conspecific populations. Multidisciplinary assays may involve a considerable amount of labour; yet this is outweighed by the total amount of information extracted and the utilisation of a suite of diverse traits. In this sense, they can be constructive in targeting research to a particular array or a combination of characters. Multidisciplinary studies are likely to be of both fundamental and practical value, especially in aquaculture, in view of the increased demands for *Artemia* cysts and biomass and the need for characterisation of strains with potentially exploitable characteristics.

The main objective of the present study was to investigate the microevolutionary changes that have taken place since the introduction of the *A. franciscana* San Francisco Bay (SFB, USA) strain into Vietnam. Temperature is a key factor, and is expected to have a bearing on the genetic architecture of *Artemia* populations in the area of the Vinh Chau saltworks, Vietnam. Therefore, the hypothesis in question deals with the response of the source population (*A. franciscana*, SFB) to a novel thermal environment. In addition, the speed of this response was tested through comparisons of early and later phases in the post-inoculation period of *Artemia* samples from Vinh Chau.

Materials and methods

Prior to its introduction, *Artemia* was not an endemic organism in the broader region of South-East Asia (Vanhaecke et al. 1987) or, specifically, in Vietnam. The first inoculation (pilot study) of *Artemia* in Vietnam took place in 1982 (Quynh and Lam 1987) on the south coast of the Mekong Delta, Vinh Chau, southern Vietnam. Culturing of brine shrimp has proven to be a profitable business, and the area is a considerable supplier of high-quality cysts (see Hoa 2002). Vietnam has a typical monsoon climate. Culturing takes place in properly managed outdoor earthen ponds and is restricted to the dry season, lasting from December to May (Baert et al. 1997), as opposed to other areas, like Brazil, where animals are cultured on a year-round basis (Camara and De Medeiros Rocha 1987). During the wet season the ponds are flooded and culturing is interrupted. Salinity levels in the ponds are kept between 80 and 120 ppt, whereas with regard to temperature (pond water) values well above 30°C are common. In sharp contrast, the *A. franciscana* population of San Francisco Bay salterns rarely experiences water temperatures $>24°C$ (typically several degrees lower) during the growing season spanning from June to October.

All cyst samples used in this study were obtained from Vinh Chau field station and from the *Artemia* Reference Center (ARC, Laboratory of Aquaculture, Ghent, Belgium). They consisted of *A. franciscana* SFB, considered as the inoculum (source) population; the Vinh Chau (VC) strain present in Vinh Chau saltfields since inoculation (1986); and strains year 1 (Y1), year 3 (Y3) and year 4 (Y4) originated after one, three and four culture seasons in Vinh Chau, respectively. Strain Y2 (after two culture seasons in Vinh Chau) was also used initially, but it was subsequently dropped from the study due to continuous and persistent crashes in the laboratory cultures.

The culture scheme in Vinh Chau is sequential in the sense that following re-inoculation with SFB, the cyst material collected at the end of each year (labelled accordingly) is used to restock ponds for the next season.

The methodological approach consisted of the comparison of genetic differences at three different levels. A number of reproductive characters were tested for interpopulation differentiation at 26°C and 30°C. Also, a battery of allozyme loci, routinely employed in *Artemia* population genetics, were screened for variation within, and differentiation between, *Artemia* populations from Vietnam. Finally, the same populations were assayed by way of restriction fragment length polymorphism (RFLP) analysis of a region of the mtDNA.

Reproductive characters

Cysts of each strain were hatched (hatching percentage was $>90\%$ for all strains) according to Sorgeloos et al. (1986), and young nauplii (350 per strain) were transferred to 1.5-l cylindroconical flasks until they reached maturity. Mating pairs (in Falcon tubes) for each strain were set up in a salinity of 80 ppt at two temperatures, 26°C and 30°C. In all stages, brine shrimps were fed on a mixed diet consisting of the alga *Dunaliella tertiolecta* Butcher and yeast (LANSY 1-PZ, INVE Aquaculture, Belgium). Five reproductive characters were daily recorded in a total of 252 animals: number of cysts per female, number of nauplii per female, total number of offspring per female, number of broods per female and number of encysted broods per female. The experiment lasted for approximately 1 month, thus allowing a considerable part of the reproductive period to elapse. Dead females were replaced only if death occurred within 3 days from the start of the experiment. In cases where death occurred past this time limit, both members of the couple were discarded. Individual dead males were replaced at any time during the experiment. Variables were routinely tested for normality and homogeneity of variances across samples. Parametric tests (two-way ANOVA, temperature and strain considered as fixed factors) were used whenever the above assumptions were met. The Bonferroni procedure (Rice 1989; Sokal and Rohlf 1995) was employed for post hoc comparisons of observed means. The statistical package STATISTICA, version 5.5 (StatSoft), was used throughout the analysis.

Allozymes

Standard horizontal starch gel electrophoresis was used. The protocols followed were those described by Murphy et al. (1996). Twenty enzyme-coding loci were scored in all strains. These were: *EST-D*, *EST-1*, *EST-4*, *6-PGDH*, *MDH-1*, *MDH-2*, *LDH*, *IDH-1*, *IDH-2*, *GOT-1*, *GOT-2*, *LAP-2*, *LAP-3*, *PGM*, *PGI*, *PEP-1*, *PEP-4*, *ME*, *SOD* and *AMY*. The TFPGA-1.3 (Miller 1997) software was used to calculate common measures of genetic variability and differentiation, as well as to construct an unweighted pair–group method with arithmetic averages (UPGMA) dendrogram of populations.

MtDNA

Following DNA extraction (Bardakci and Skibinski 1994), samples were loaded on an automatic thermocycler (Hybaid Omnigene) for amplification of a 2,963-bp-long mtDNA target sequence. The amplified region contained the following genes: part of ND5, ND4, ND6, various tRNAs and part of Cytb. The primers AHND5 and AL1Cytb were supplied from Amersham Pharmacia Biotech and diluted according to manufacturer's instructions to give a final concentration of 30 ng μl^{-1}.

For each amplification, the total reaction volume of 25 μl consisted of 2.5 μl 10× reaction buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, Advanced Biotechnologies), 4 μl dNTPs (1 μl of each base at 100 μM, Promega), 0.8 μl primer (0.4 μl each at 30 ng μl^{-1}), 0.15 μl *Taq* DNA polymerase (ABgene), 1 μl template DNA (25 ng μl^{-1}) and 16.55 μl dH_2O. Each of the 30 amplification cycles consisted of 1 min denaturation at 94°C, 1 min annealing at 60°C, 3 min 30 s extension at 72°C and a final extension of 10 min at 72°C. Eight restriction endonucleases (*Alu*I, *Ava*I, *Eco*RI, *Hae*III, *Hind*III, *Hinf*I, *Rsa*I, *Xba*I) were employed to assess variation in the amplified region. Digested products were electrophoresed on 5% vertical polyacrylamide gels and visualised by silver staining. Data (fragment approach) were analysed by REAP, version 4.0 (McElroy et al. 1992), and NTSYS, version 1.2 (Applied Biostatistics).

Results

Differences in reproductive traits

There is ample evidence of divergence in reproductive traits between the source *Artemia franciscana* SFB population and those found in Vietnam, as well as between the year samples of the latter. Temperature appears to be invariably involved in the observed differentiation between strains, either as a single factor or interactively with respect to the particular strain. Results of all five analyses of variance are shown in Table 1. Out of the five reproductive variables analysed, only the number of encysted broods per female did not yield a significant temperature×strain interaction term ($P = 0.34$). The responses of the five reproductive characters for all strains across temperatures are shown in Fig. 1a–e. The five different fecundity traits assayed constitute a generalised index that could be collectively regarded as the reproductive output. This index seems to be a decreasing function of temperature; in all cases, reproduction is lower at 30°C, and this trend is statistically significant (P-values range from 0.002 to $< 10^{-6}$, see Table 1). The only exception occurs with the VC strain, which shows either a marked increase (post hoc tests, P-values $< 10^{-3}$) of its reproductive output (as expressed by the number of nauplii, the number of total offspring and the number of broods per female, see Fig. 1b, c, d) at 30°C or a non-significant decline ($P > 0.99$ for the number of cysts and encysted broods per female, see Fig. 1a, e) at 26°C. For the other strains, differences in reproductive output across temperatures (as determined by post hoc comparisons) occur for SFB ($P = 0.017$ for the total offspring), Y3 (except for the number of cysts) and Y4 ($P < 10^{-3}$). It is also worth noting that strain Y1 shows remarkable stability for all reproductive variables between temperatures.

Table 1 *Artemia franciscana*. Results of two-way analyses of variance for five reproductive characters

Character	Source	*df*	F_S	P-value
Cysts/♀	Temperature	1	27.28	$< 10^{-6}$
	Strain	4	9.89	$< 10^{-6}$
	Interaction	4	2.67	0.032
	Error	242		
Nauplii/♀	Temperature	1	17.34	$< 10^{-3}$
	Strain	4	16.99	$< 10^{-3}$
	Interaction	4	16.57	$< 10^{-3}$
	Error	242		
Total offspring/♀	Temperature	1	80.67	$< 10^{-6}$
	Strain	4	22.51	$< 10^{-6}$
	Interaction	4	32.15	$< 10^{-6}$
	Error	242		
Broods/♀	Temperature	1	16.15	$< 10^{-3}$
	Strain	4	27.89	$< 10^{-6}$
	Interaction	4	41.56	$< 10^{-6}$
	Error	242		
Encysted broods/♀	Temperature	1	9.45	0.002
	Strain	4	13.52	$< 10^{-6}$
	Interaction	4	1.13	0.34
	Error	242		

It is therefore clear that differences in temperature have a variable effect on the reproductive traits of the investigated strains (interaction). Yet, this is not true for the number of encysted broods, since temperature variation affects all strains equally.

Considering within-treatment variability, the different strains seem to form a rather homogeneous group at the temperature of 26°C. Especially for the number of nauplii per female, there are no significant differences between strains. For the remaining traits, post hoc tests show that strains Y3 and Y4 cluster together, while separately ($P < 0.05$) from all the rest. In contrast, within 30°C the VC strain is either excluded from the group of the remaining strains ($P < 10^{-6}$ for nauplii, total offspring and broods per female), or it clusters with the different year samples (cysts and encysted broods per female). With the exception of the number of encysted broods, strains SFB and VC are members of different sets and, therefore, markedly different ($P < 10^{-6}$). An example of the apparent differentiation between strains at 30°C for the five variables analysed is given in Fig. 2. In the dendrograms shown, strains have been clustered, via the UPGMA algorithm, over Euclidean pairwise distances for each variable. The resulting topology is consistent with the sets of homogeneous groups revealed by the post hoc analysis.

Partitioning of the components of variance can be achieved by considering the strain effect as a random factor (two-way ANOVA, mixed model). The rationale here is that the different populations of *A. franciscana* investigated could arguably be thought of as a random sample of other such populations. In fact, treating the different strains as fixed effects in the current study may be justified on the grounds of the particular strains investigated. However, this practice is not entirely intuitive with regard to our hypothesis as the genetic background is also of special interest. Therefore, using a mixed-model approach (which does not

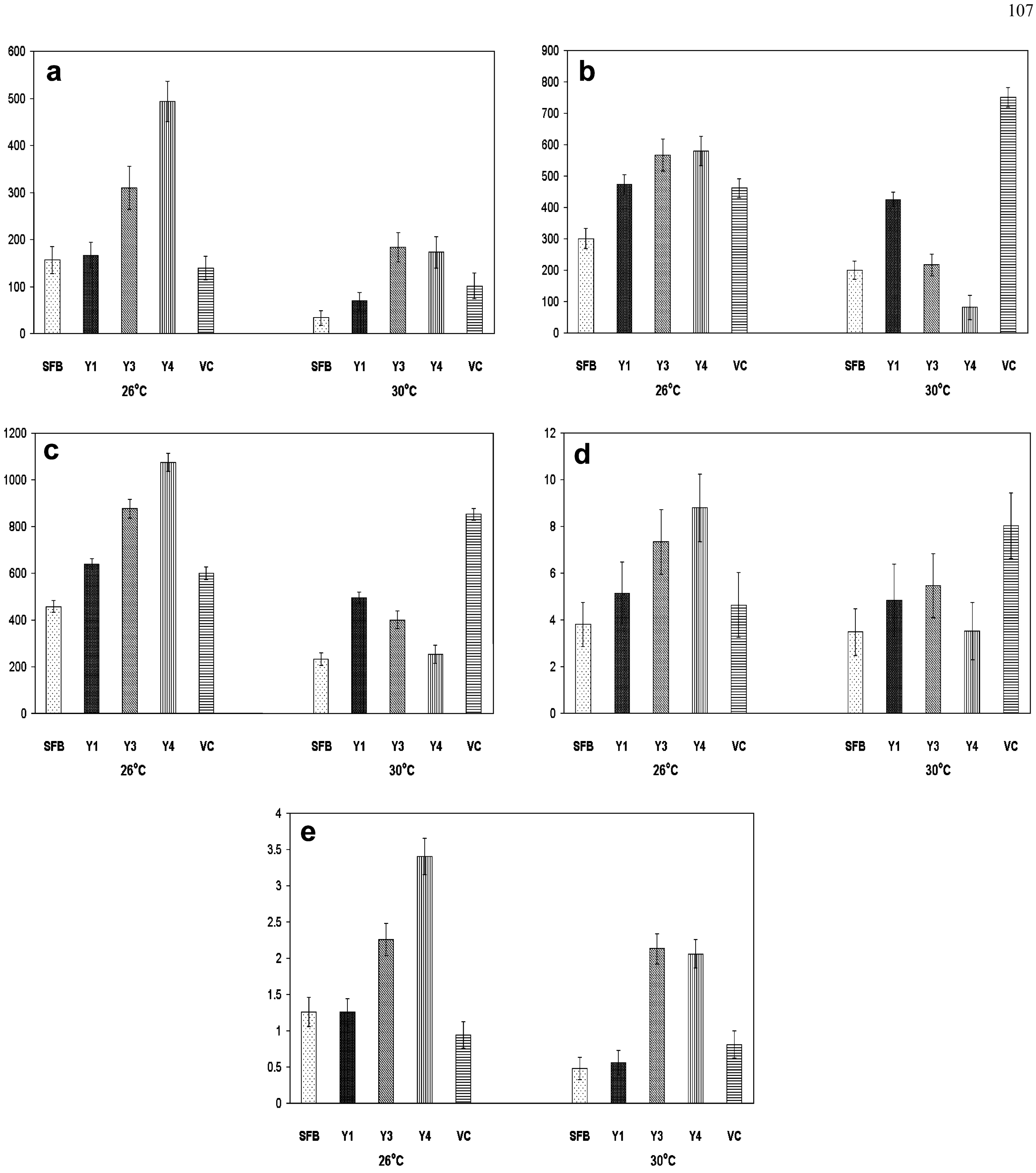
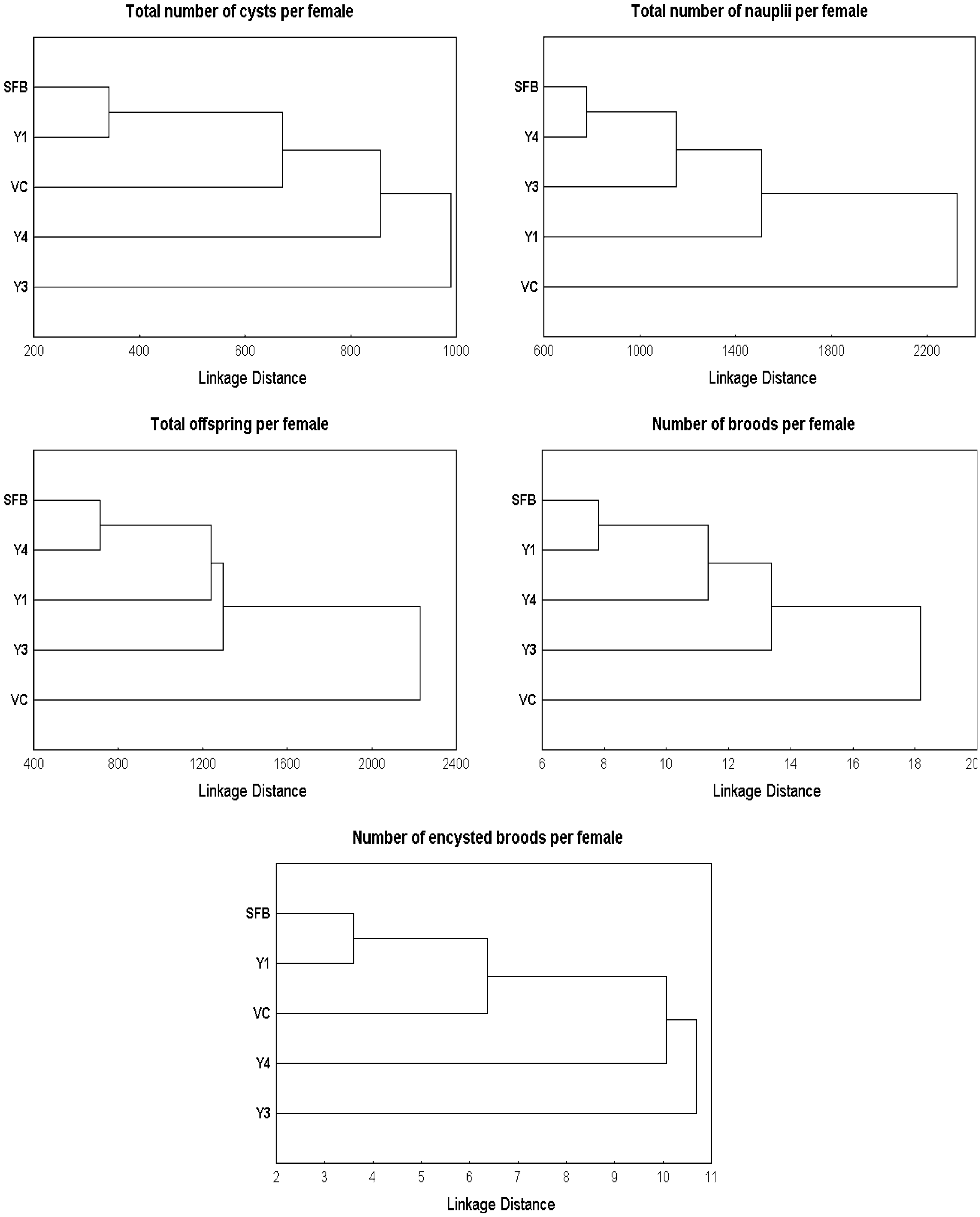


Fig. 1a–e *Artemia franciscana*. Reproductive response profiles for all strains (*SFB* San Francisco Bay; *VC* Vinh Chau; *Y1*, *Y3*, *Y4* years 1–4 after inoculation) at 26°C and 30°C. Shown are mean values (±standard deviations): **a** cysts/♀, **b** nauplii/♀, **c** total offspring/♀, **d** broods/♀, **e** encysted broods/♀

alter the overall results), the different variance components for each reproductive trait are broken up to an error term, pertinent to unexplained variation, a strain term and an interaction (temperature×strain) term, considered as a random effect. The obtained partitioning is shown in Table 2. The magnitude of the unexplained variation ranges from 37.6% for the number of broods to 82.2% for the number of cysts. The lowest pure genetic component (strain term) that is present occurs for the number of nauplii (0.5%), whereas all components of variance accounted for by the interaction term (except encysted broods) are statistically significant (Satterthwaite's method of denominator synthesis implemented in STATISTICA, see Table 2).



Total number of cysts per female

Total number of nauplii per female

Total offspring per female

Number of broods per female

Number of encysted broods per female

Fig. 2 *Artemia franciscana*. UPGMA (unweighted pair-group method using arithmetic averages) clustering of strains over Euclidean pairwise distances for each reproductive variable at 30°C

Allozyme differentiation

Twenty enzyme-coding loci were successfully scored. Two loci, *PGM* and to a greater extent *PEP-1*, produced strong satellite banding; this has also been noted by several other workers (e.g. Abreu-Grobois 1983; Pilla 1992). Eleven enzyme systems (*EST-D*, *EST-1*, *EST-4*, *MDH-1*, *MDH-2*, *LDH*, *LAP-2*, *LAP-3*, *ME*, *SOD*, *AMY*) were consistently fixed across strains for the same allele. Loci *GOT-1* and *PEP-4* exhibited, occasionally, a faster and a slower migrating allele, respectively, both at frequencies <0.05. Allele frequencies together with genetic variability estimates and probability values for conformity to Hardy–Weinberg (H–W) expectations in all strains are shown in Table 3. For the H–W tests, the type-I error rate was controlled (Bonferroni correction) within each temperature. Standard errors (not shown) for values of heterozygosity and mean number of alleles per locus were in the range of 0.028–0.042 and 0.1–0.2, respectively.

Overall, nine loci displayed polymorphism at 26°C. The locus *PEP-4* was only variable in the SFB strain. The same strain displayed all four *PGM* alleles. Significant H–W disequilibria, possibly due to selection against heterozygotes, were observed for the locus *GOT-1* in the Y3 strain, as well as in most strains for the locus *PGM* (see Table 3). Heterozygosity values were relatively invariable within this temperature as were the percentage of polymorphic loci and the mean number of alleles per locus. Patterns of polymorphism at 30°C were generally similar to those at 26°C, with the exceptions of marked H–W deviations at the *PEP-1* locus and the absence of polymorphism at *PEP-4*. Also, heterozygosity values were higher compared with those at 26°C, however not significantly so.

Exact tests (Fisher's R×C) for population differentiation using a Markov chain Monte Carlo approach (Raymond and Rousset 1995) are shown in Table 4. Also shown are global tests (Fisher's combined probability test, Sokal and Rohlf 1995) over loci for determination of overall significance in each case as well as treatment-wise. There is no substantial differentiation between strains at 26°C. In contrast, significant heterogeneity in allele frequencies is observed at 30°C, as well as between temperature groups. This is also evident in the topology of the UPGMA dendrogram (Fig. 3) based on Nei's (1972) genetic distance between strains. Temperature groups are clustered separately; at 26°C there seems to be less differentiation compared with the more heterogeneous cluster at 30°C, from which the VC strain branches off quite distinctly from the remaining strains.

Table 2 *Artemia franciscana*. Partitioning of the variance components for the two-way ANOVA for each reproductive character (****P* is statistically significant at the 0.001 level)

Character	Error	Strain	Interaction
Cysts/♀	82.2%	12.2%	5.6%***
Nauplii/♀	60.7%	0.5%	38.8%***
Total offspring/♀	43.9%	–	56.1%***
Broods/♀	37.6%	–	62.4%***
Encysted broods/♀	79.5%	20.1%	0.4%

MtDNA divergence

The mtDNA analyses between the two temperature groups show no significant differences; therefore, only results from the 26°C data set are presented below. Enzymes *Ava*I, *Eco*RI and *Hind*III detected a lack of polymorphism across strains. These enzymes displayed three, two and two fragments, respectively. A total of 83 fragments were surveyed in the mtDNA target sequence. As the average number of bases examined was 224.54 and the *A. franciscana* mitochondrial genome is estimated to be 15,822 nucleotides long (Valverde et al. 1994), 1.42% of the *Artemia* mtDNA was screened. Homology of fragments was established through side-by-side gel comparisons and also through comparisons of restriction patterns to those generated by the Gene Runner computer package (version 3.0, Hastings Software). Examples of restriction profiles are shown in Fig. 4.

A total of 11 mtDNA composite genotypes were identified. Their frequency distribution was homogeneous across strains (chi-squared test, $P=0.41$, Roff and Bentzen 1989). Haplotype frequency estimates together with haplotype and nucleotide diversity values are given in Table 5. Only one composite genotype (H1) occurred in all strains and appeared to be increasing in frequency through the different year classes leading to VC. This pattern of increase was not significant ($P=0.09$) when tested through a Monte Carlo approach (Roff and Bentzen 1989). The next most common haplotype was H5, observed in SFB and the year classes. There were eight private haplotypes (H3, H4, H6–11) detected among the SFB, Y1, Y3 and VC strains. Of these, the Y1 strain displayed the most (H7–10), the SFB strain two (H3, H4), while each of the Y3 and VC strains displayed one (H6 and H11, respectively). Most composite genotypes (six) were observed in the Y1 strain, followed by SFB (five), Y3 and Y4 (three each) and finally VC with only two. This is reflected, as one might expect, in a concomitant pattern of decrease at haplotype diversity levels, with the VC strain showing the lowest estimate (see Table 5). On the other hand, nucleotide diversity appears to be increasing in the Vietnamese strains, albeit with less consistency. The highest degree of sequence divergence exists between H3 and H6 (0.0469 ± 0.019), which are both unique: H3 to SFB and H6 to VC. The average estimate of nucleotide divergence between all pairs of haplotypes is 0.0147. The divergence matrix between haplotypes (not shown) was used for clustering by the UPGMA method. Three tied trees were obtained, differing in the relative positions of H1, H2, H4 and H7. One of these dendrograms is shown in Fig. 5. H6 (private for VC) appears well differentiated from all the rest. The latter form two groups: the first is represented by

Table 3 *Artemia franciscana*. Allele frequencies and genetic variability estimates in all strains across temperatures (only polymorphic loci are shown) [H_o observed heterozygosity; H_e expected heterozygosity (unbiased estimate, Nei 1978);*%P* percentage of polymorphic loci (99% criterion); *m.n.a.* mean number of alleles per locus; *P* probability of conformity to Hardy–Weinberg (H–W) proportions (exact test); –, not observed]. Values in **bold** indicate significant H–W deviations (Bonferroni correction) within each temperature treatment

	26°C					30°C				
	SFB	Y1	Y3	Y4	VC	SFB	Y1	Y3	Y4	VC
*6-PGDH*98*	0.375	0.354	0.490	0.365	0.427	0.459	0.417	0.354	0.365	0.469
*6-PGDH*100*	0.625	0.646	0.510	0.635	0.573	0.541	0.583	0.646	0.635	0.531
H_o	0.625	0.458	0.437	0.562	0.520	0.541	0.458	0.541	0.562	0.562
H_e	0.473	0.462	0.505	0.468	0.494	0.501	0.491	0.462	0.468	0.503
P	0.027	0.995	0.405	0.220	0.779	0.769	0.767	0.345	0.217	0.554
*IDH-1*98*	0.135	0.178	0.209	0.072	0.198	0.062	0.062	0.020	0.052	0.042
*IDH-1*100*	0.865	0.822	0.791	0.928	0.802	0.938	0.938	0.980	0.948	0.958
H_o	0.187	0.270	0.250	0.062	0.270	0.083	0.125	0.041	0.104	0.041
H_e	0.236	0.294	0.333	0.136	0.320	0.118	0.118	0.041	0.099	0.080
P	0.191	0.616	0.088	0.011	0.352	0.154	0.995	0.995	0.995	0.063
*IDH-2*100*	0.980	0.917	0.896	0.959	0.948	0.948	0.927	0.959	0.896	0.917
*IDH-2*102*	0.020	0.083	0.104	0.041	0.052	0.052	0.073	0.041	0.104	0.083
H_o	0.041	0.166	0.166	0.083	0.104	0.104	0.145	0.083	0.166	0.125
H_e	0.041	0.154	0.188	0.080	0.099	0.099	0.136	0.080	0.188	0.154
P	0.995	0.995	0.404	0.995	0.995	0.995	0.995	0.995	0.406	0.273
*GOT-1*100*	1.000	1.000	0.959	0.980	0.980	0.980	0.959	0.980	1.000	1.000
*GOT-1*102*	–	–	0.041	0.020	0.020	0.020	0.041	0.020	–	–
H_o			0.000	0.000	0.000	0.000	0.000	0.000		
H_e			0.080	0.041	0.041	0.041	0.080	0.041		
P			**$<10^{-3}$**	0.010	0.009	0.007	**$<10^{-3}$**	0.010		
*GOT-2*98*	–	–	0.010	0.083	–	0.125	0.115	0.052	–	–
*GOT-2*100*	1.000	1.000	0.990	0.917	1.000	0.875	0.885	0.948	1.000	1.000
H_o			0.020	0.083		0.166	0.187	0.062		
H_e			0.020	0.154		0.221	0.205	0.099		
P			0.995	0.022		0.141	0.477	0.098		
*PGM*96*	0.031	–	–	–	–	0.010	–	0.020	–	0.073
*PGM*98*	0.135	0.104	0.135	0.125	0.167	0.230	0.104	0.188	0.156	0.125
*PGM*100*	0.688	0.709	0.740	0.750	0.646	0.677	0.750	0.667	0.802	0.792
*PGM*102*	0.146	0.187	0.125	0.125	0.187	0.083	0.146	0.125	0.042	0.010
H_o	0.250	0.333	0.208	0.125	0.208	0.312	0.166	0.166	0.270	0.208
H_e	0.491	0.457	0.423	0.410	0.525	0.487	0.409	0.509	0.334	0.355
P	**$<10^{-5}$**	0.013	**$<10^{-5}$**	**$<10^{-5}$**	**$<10^{-5}$**	**$<10^{-3}$**	**$<10^{-5}$**	**$<10^{-5}$**	0.062	**0.002**
*PGI*98*	0.365	0.292	0.292	0.438	0.385	0.344	0.250	0.385	0.375	0.396
*PGI*100*	0.635	0.708	0.708	0.562	0.615	0.656	0.750	0.615	0.625	0.604
H_o	0.437	0.458	0.333	0.458	0.520	0.479	0.291	0.437	0.541	0.583
H_e	0.468	0.417	0.417	0.497	0.478	0.455	0.378	0.478	0.473	0.483
P	0.756	0.727	0.175	0.777	0.759	0.757	0.133	0.556	0.371	0.222
*PEP-1*98*	0.041	0.020	0.020	0.094	0.041	0.230	0.135	0.292	0.417	0.719
*PEP-1*100*	0.959	0.980	0.980	0.906	0.959	0.770	0.865	0.708	0.583	0.281
H_o	0.083	0.041	0.041	0.104	0.083	0.291	0.104	0.041	0.333	0.229
H_e	0.080	0.041	0.041	0.171	0.080	0.357	0.236	0.417	0.491	0.408
P	0.995	0.995	0.995	0.041	0.995	0.233	0.002	**$<10^{-5}$**	0.041	**0.002**
*PEP-4*100*	0.969	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
*PEP-4*98*	0.031	–	–	–	–	–	–	–	–	–
H_o	0.020									
H_e	0.061									
P	0.030									
m.n.a.	1.45	1.35	1.45	1.45	1.4	1.5	1.45	1.5	1.35	1.4
%*P*	35	30	35	40	30	40	40	35	30	30
$\hat{H}_o$	0.078	0.068	0.062	0.071	0.080	0.103	0.071	0.068	0.103	0.087
$\hat{H}_e$	0.092	0.079	0.089	0.097	0.091	0.118	0.106	0.104	0.109	0.096

H3 (unique to SFB) and the second by all the remaining haplotypes.

Overall mean pairwise estimates of mtDNA nucleotide sequence divergence (d_A) between strains, corrected for interpopulation diversity (d_{XY}), are shown in Table 6. Minimal nucleotide divergence is observed between strains. This is also confirmed by the partitioning of the total interpopulation diversity: N_{ST} (Lynch and Crease 1990) equals 0.0266, meaning that 2.66% of nucleotide diversity is distributed between strains. The pairwise nucleotide divergence values (not shown) were used as an input matrix for the construction of a phylogenetic tree for all strains (Fig. 6). With regard to the negative branch lengths obtained, it has to be noted that these are due to negative values for some pairwise comparisons of nucleotide divergence. The true value of

Table 4 *Artemia franciscana.* Exact tests for allele frequency homogeneity within and between temperature treatments (strain abbreviations, see Fig. 1 legend)

Level	Loci	Significant comparison of strains	Combined *P*-value over loci	Combined *P*-value for treatment
Within 26°C	*PEP-1*	VC vs. Y4	> 0.99	> 0.980
	PEP-1	Y1 vs. Y4	> 0.99	
	PEP-1	Y3 vs. Y4	> 0.97	
Within 30°C	*GOT-2*, *PGM*,*PEP-1*	SFB vs. VC	0.009	0.015
	PGM	SFB vs. Y1	> 0.99	
	GOT-2, *PEP-1*	SFB vs. Y4	0.768	
	GOT-2, *PGM*,*PEP-1*	VC vs. Y1	0.003	
	PEP-1	VC vs. Y3	0.189	
	PEP-1	VC vs. Y4	0.528	
	PGI, *PEP-1*	Y1 vs. Y3	0.923	
	GOT-2, *PGM*,*PGI*, *PEP-1*	Y1 vs. Y4	0.096	
Between	*GOT-2*, *PGM*,*PEP-1*			0.026

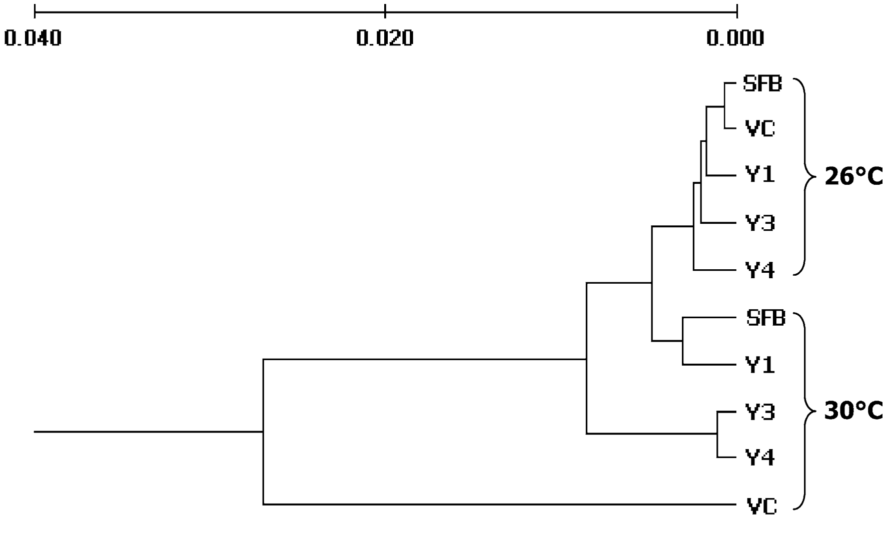
nucleotide divergence may be positive but small. In these cases, the estimate of the value is about as likely to be below as above the true value. In genetic terms, this is ultimately derived from the fact that, given the current level of differentiation, haplotypes are more related between than within strains. Therefore, polymorphism has not yet been converted to divergence per se. Two distinct groups are evident from the dendrogram in Fig. 6. The SFB strain is clearly differentiated from the group consisting of the Vietnamese strains. Within the latter, VC markedly branches off the cluster of year classes. Also interesting is the separation of Y1 from both Y3 and Y4.

Discussion

Over the last two decades, a rather specific molecular arsenal has dominated most of the research on the evolutionary relationships between species and the patterns of intraspecific differentiation in the genus of *Artemia*. The use of allozyme markers, reproductive traits and life-span characteristics and, relatively recently, mtDNA (see also Abatzopoulos et al. 2002b, and references therein) have proven a fairly edifying line of investigation that provided sufficient discrimination between species and/or populations, as well as valuable insights into population structure and physiological adaptation.

Considering the importance of *Artemia* in aquaculture, monitoring of strains introduced to different areas worldwide has acquired particular weight, not only for the future success and further development of inoculation programs, but also for the advancement of certain issues related to tolerance, life strategies in and response to different environments and others. Divergence in survivorship at temperatures > 30°C has previously been demonstrated between *A. franciscana*, SFB populations and those in Macau, Brazil and Barotac Nuevo, Philippines, derived from inoculated SFB cysts (see Lenz and Browne 1991, and references therein). In similar studies, Wear and Haslett (1986) and Wear et al. (1986) reported that the almost exclusive ovoviviparity of a New Zealand population of *A. franciscana* (inoculated in the early 1950s) was due to divergence brought about by year-round constant salinity and temperature. Laboratory and field data, however, show that there is no consensus over the factors thought to be responsible for the observed patterns of reproductive ecology in natural

Fig. 3 *Artemia franciscana.* UPGMA dendrogram of Nei's (1972) genetic distance between strains at 26°C and 30°C. Bootstrap values of node support out of 1,000 permutations range between 60% and 80%



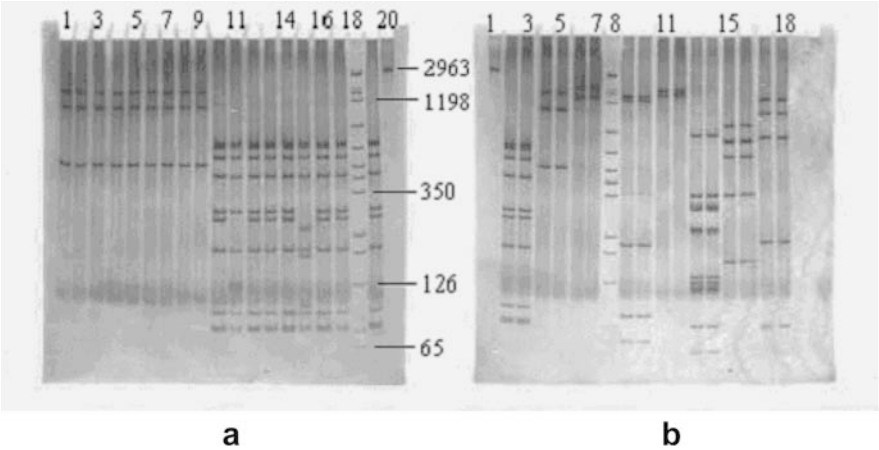


Fig. 4a, b *Artemia franciscana*. Examples of digestion profiles. **a** Strain SFB—lanes 1–9 digestion with *Ava*I; lanes 10–17 and 19 digestion with *Alu*I; lane 18 pGEM-3 DNA marker; lane 20 undigested control (2,963 bp). **b** Strain VC, digestion of two individuals with all eight endonucleases—lanes 2–3 *Alu*I; lanes 4–5 *Ava*I; lanes 6–7 *EcoR*I; lanes 9–10 *Hae*III; lanes 11–12 *Hind*III; lanes 13–14 *Hinf*I; lanes 15–16 *Rsa*I; lanes 17–18 *Xba*I; lane 8 pGEM-3 DNA marker; lane 1 undigested control (2,963 bp)

Table 5 *Artemia franciscana*. Mitochondrial DNA variability estimates in all strains (composite genotypes are denoted by lower case letters, each one corresponding to a restriction endonuclease pattern in the order *Alu*I,*Ava*I, *EcoR*I, *Hae*III,*Hind*III, *Hinf*I, *Rsa*I,*Xba*I)

Haplotype	Composite genotype	SFB	Y1	Y3	Y4	VC
1	aaaaaaaa	0.7692	0.7619	0.7857	0.8571	0.9473
2	aaabaaaa	0.0384			0.0714	
3	caacafca	0.1153				
4	aaadaaaa	0.0384				
5	baaaaaab	0.0384	0.0476	0.1428	0.0714	
6	daaaaebc					0.0526
7	aaaeaaaa		0.0476			
8	aaaaadab		0.0476			
9	aaaaaaac		0.0476			
10	aaaaacaa		0.0476			
11	aaaaabaa			0.0714		
Haplotype diversity (*h*)		0.4062	0.4286	0.3846	0.2747	0.1053
± SE		0.11	0.13	0.14	0.14	0.09
Average (± SE)				0.3199 (0.003)		
% Nucleotide diversity (π)		0.65	0.15	0.16	0.10	0.34
% Average (± SE)				0.29 (10^{-6})		

and artificial brine shrimp populations. In this study, we have tried to assess overall differentiation from a multidisciplinary angle, focusing mainly on different markers showing congruent profiles and less on individual discriminating factors.

With regard to the five reproductive traits analysed, substantial evidence has been gathered on divergence between strains. The overall differentiation appears to be a product of the whole set of reproductive variables, with exception of the encysted broods per female (see Table 1). The VC strain consistently outperformed strains SFB and Y1 in terms of reproductive output at the temperature of 30°C. In all cases, the better performance of VC was significant, a strong indication of

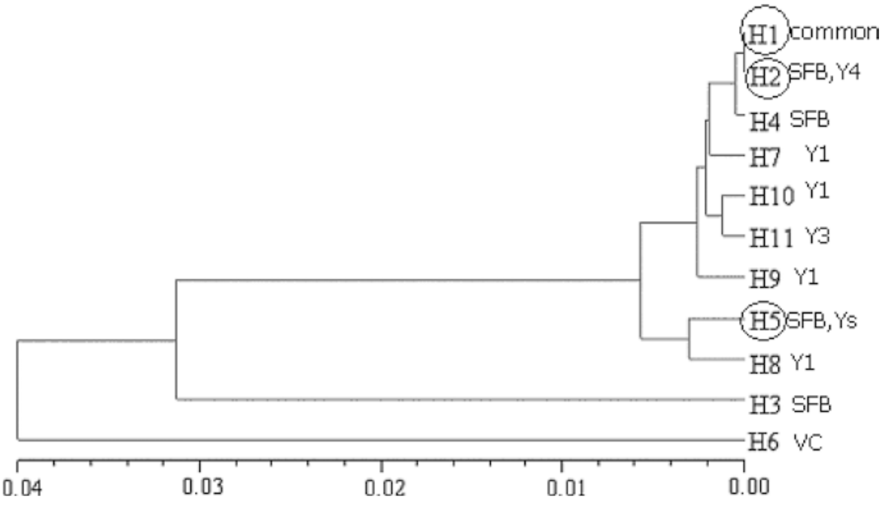


Fig. 5 *Artemia franciscana*. UPGMA dendrogram of nucleotide divergence for mtDNA composite genotypes in the strains studied. *Circles* indicate non-private haplotypes (*H1–H11* haplotypes 1–11, see Table 5)

Table 6 *Artemia franciscana.* Overall mean nucleotide diversity and nucleotide divergence values between strains

	Nucleotide diversity (d_{XY})	Nucleotide divergence (d_A)
Mean ($\pm$SE)	0.002923 ($\pm 10^{-6}$)	0.00008 ($\pm 10^{-7}$)
Minimum	0.001265	0.000045
Maximum	0.005198	0.000287

thermal adaptation. This is further supported by the observation that Y1 achieved second place consistently. Over the five reproductive characteristics examined, the dominant pattern, in terms of overall reproductive output at 30°C, was VC > Y1 > SFB. Therefore, clear-cut differentiation exists between strains, which is already manifest after just 1 year (Y1) of culturing in Vinh Chau saltfields. Moreover, at the same temperature VC is also significantly different from Y1 (for nauplii, total offspring and broods per female, $P < 10^{-5}$), which, in turn, differs markedly from SFB ($10^{-4} < P < 0.041$). The fact that the VC strain displays distinctive profiles for fitness-related attributes at increased temperature, as a result of thermal adaptation, could be further supported by the work of Hoa (2002), who demonstrated significantly higher survival for the VC strain at 30°C and 34°C compared with *A. franciscana* SFB. More interestingly, Clegg et al. (2000, 2001) and Frankenberg et al. (2000) studied components of the heat-shock response (an induced character) in the same strains, and provided evidence for increased thermotolerance in cysts and adults of Vietnamese origin. It is therefore quite conceivable, as gauged by our results as well as those of other workers, that variation in a number of characters between VC and SFB strains has a firm genetic basis. The deviant patterns observed for strains Y3 and Y4 at both temperatures could be accounted for by reduced sample sizes or favourable environmental conditions during the respective years of harvesting.

The absence of interaction for the variable encysted broods indicated that all strains are similarly affected by temperature differences (see Fig. 1e). This may be due to the fact that differences between strains for this trait could not be detected over the range of investigated temperatures. It is possible that a more stressful environment (e.g. 34°C) would have triggered the required stimuli, resulting in strain discrimination due to temperature. In other words, it would have probably produced an interaction effect for the respective trait as well. Examination of Fig. 1e reveals that the decrease in the number of encysted broods from 26°C to 30°C for the VC and Y3 strains is very small. Presumably, more complex genetic interactions and regulatory control are involved in the encystment process; indications in favour of this supposition come from inspection of the variance components in Table 2. Among the five reproductive variables, the number of encysted broods occupies the highest component (20.1%) of the total variance attributable to the genetic makeup (strain factor). Therefore, encystment seems to be a character well canalised into the genetic architecture of a species. It is not surprising that a considerable portion (12.2%) of the total variance for the number of cysts per female is also due to the genetic constitution. This may be partly explained from evidence (Vanhaecke et al. 1983) that encystment is energetically more expensive compared with ovoviviparity. Thus, characters related to such multifaceted processes as energy allocation and balance are arguably expected to be under more strict genetic control. Our data suggest that a measurable genetic factor is involved in encystment. This is in agreement with the results of Browne and Spencer (1987) on variable encystment rates among clones within an asexual population and those of Gajardo and Beardmore (1989) on the correlation between female heterozygosity and level of encystment. On the other hand, Browne et al. (1984, 2002) have reported quite different magnitudes and patterns of genetic components for a number of reproductive and lifespan characteristics, including encystment. Genetic components of demonstrable magnitude offer the possibility of manipulation through

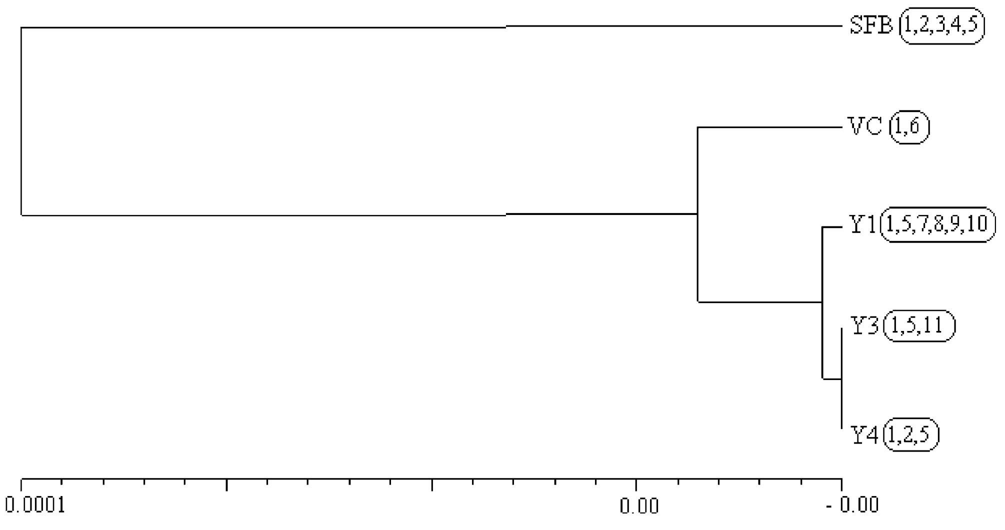


Fig. 6 *Artemia franciscana.* UPGMA dendrogram of mtDNA sequence divergence for the strains studied. *Numbers* indicate haplotype content and correspond to the haplotype list in Table 5

selection, especially for commodities as important for aquaculture as cysts. It has to be recognised, however, that in this study the five reproductive variables were examined over two levels. Inclusion of additional factors is certainly needed in order to reach more tenable inferences by eliminating as much as possible of the unexplained variation (error term in Table 2) in investigated traits.

Another possible explanation for the absence of interaction for the number of encysted broods is phenotypic plasticity. It may well be that the examined temperatures fall within the genotypic reaction norm of strains with respect to encystment, although this does not apply for the remaining variables. Berthélémy-Okazaki and Hedgecock (1987) studied the effects of several physical and biological factors on cyst formation and concluded that no single factor could sufficiently explain the switch in reproductive mode. The indications we have obtained are similar to those of other authors, who have developed a working hypothesis based on induction of stress.

The possibility that the observed differentiation in reproductive profiles may be due to founder effects (see Winkler et al. 1999) of the different year class strains (Y1, Y3, Y4) can be ruled out for two reasons. First, the cyst material used for inoculation in each consecutive year amounts to several hundred grams. On the average, 250,000–280,000 nauplii are hatched from 1 g of *Artemia* SFB cysts. This ensures that there are no sampling effects and most of the gene pool is represented in the next year. Second, the levels of genetic variation, as determined allozymically, are in good agreement with previous studies (Abreu-Grobois 1983; Pilla 1992; Thomas 1995). Mean values for expected heterozygosity and percentage of polymorphic loci for *A. franciscana* populations are 0.09 (range 0.02–0.14) and 30.3 (range 9–50), respectively. The genetic variability estimates obtained in this study fall well within the above ranges. Moreover, tests (Mann–Whitney, not shown) of difference in heterozygosity values between strains were not significant, despite the historical correlation of their heterozygosities (Nei 1987). Regarding the abundance of data on *A. franciscana* compared with other bisexual species of the genus, its high levels of polymorphism are further confirmed and validate its use for aquaculture purposes worldwide. The absence of genetic impoverishment in the investigated strains attests to the evolutionary potential of *A. franciscana* as a genetically rich species, able to respond to novel environments. This is of outmost importance for the aquaculture industry, since maintaining variability is one of the prime concerns of any inoculation scheme. Towards this direction, more sensitive genetic markers like microsatellite DNA (Reilly et al. 1999) or RAPDs (Koh et al. 1999) may shed additional light on the genetic composition of the reared strains.

The allozyme survey has provided evidence of considerable genetic differences between temperature groups. Gauged by the results on heterogeneity of allele frequencies, patterns of divergence are not generated by the actual genetic composition of the examined strains (no fixed allelic differences, only one unique allele at *PEP-4*, see Table 3), but rather from shifts of the allele frequency distribution at polymorphic loci of a common gene pool. Thus, starting from the SFB source population, overall polymorphism is broken up with time into a number of genetic variability profiles, leading through the successive year classes to the VC strain. The genetic differentiation on the whole is not of great magnitude, especially considering that it is almost invariably supported by a limited number of polymorphic loci (see Table 4). However, this is the first step of microevolutionary changes leading eventually to geographic differentiation and progressive adaptation to new environments. At the temperature of 26°C, allele frequencies are largely homogeneous, whereas, at 30°C, as well as between temperatures, pronounced differentiation is evident (see Table 4). In particular, the VC strain (30°C) is markedly divergent from SFB and Y1 ($0.003 < P < 0.009$). This pattern is impressively consistent with that observed for reproductive traits. In this respect, the dendrogram in Fig. 3 provides further confirmation of the net result of the sequential culturing used and, at the same time, is a firm indication that selective forces are more likely candidates for the apparent differentiation as opposed to genetic drift, despite the relatively low values of node support.

Because of the short time-scale involved, meta-analytical tools (combining probabilities) of increased power were employed in order to detect differences between strains. The microevolutionary changes that have taken place since the inoculation of *Artemia* SFB in Vietnam have produced genomic partitions discernible by allozyme markers and comparable to those found at an initial stage of divergence between geographic populations in nature.

Unlike allozymes, strong indications of reduction in mtDNA gene diversity were obtained in the Vietnamese strains. Examination of Table 5 shows a gradual loss of haplotype diversity leading to the VC strain. Despite the absence of heterogeneity in haplotype frequencies between strains, there are two distinctive features that deserve special reference. The first is the largely heterogeneous haplotype content of strains SFB and Y1; among the nine composite genotypes detected within these strains only two of them are common. Strain Y1 displays the highest number of haplotypes (six), four of which are private. Estimates of gene diversity obtained from mtDNA are expected to exhibit a larger variance than similar estimates based on a large number of nuclear loci (Nei 1987). Despite the current debate over mtDNA transmission genetics (see Rokas et al. 2003, and references therein), the occurrence of maternal and clonal inheritance reduce its effective population size to only about one-fourth that of nuclear genes. Therefore, population subdivision is not expected to have the same effect on the geographic distribution of variability in nuclear and mitochondrial genomes (Birky et al. 1983).

As a consequence, a population can be effectively more subdivided for organelle genes, while the nuclear genes are indicative of panmixia (Crease et al. 1990).

Although neutrality is assumed for mtDNA polymorphisms, selection for particular haplotypes cannot be completely ruled out. This is indicated by the second feature of the analysis regarding the VC strain, which appears to be close to a monomorphic state: it displays the least haplotypes (H1, H6), one of which (H1, the global haplotype) attains its highest frequency (0.9473) within VC, albeit in a non-significant ($P = 0.09$) manner. In addition, H6 shows the highest level of mean nucleotide divergence of all remaining haplotypes (3.646%), followed by H3 (3.078%, unique to SFB). The speed by which the established VC strain has approached near fixation for the most prevalent haplotype is suggestive of strong selective pressures on the mtDNA genome. Purifying selection through differential survivorship could presumably be accounted for by the very short coalescence times of several alleles (e.g. H2, H5, H11), which are quickly removed because of their potentially deleterious effects. However, this is currently open to speculation, and larger sample sizes are certainly needed before inferences based on selective pressures can safely be put forward, especially considering that most private haplotypes were represented by as few as one to three individuals. As a result of that, we were unable to conduct a meaningful test for differential performance of the observed haplotypes. Although recent research and re-evaluation of patterns of mtDNA evolution have brought the assumed neutrality of mtDNA polymorphisms into question (Gerber et al. 2001; Gemmell et al. 2004), it might well be that the limited variance of the observed mtDNA diversity is due to mutation–drift equilibrium rather than any form of selection. More powerful data, like sequencing, could probably detect subtle differences between mitochondrial haplotypes against divergent nuclear backgrounds, favouring co-evolved mitochondrial and nuclear genomes. This is especially true considering the increase in nucleotide diversity of VC compared to the year classes (see Table 5), as well as the maximum degree of sequence divergence (0.0469) between H3 and H6, unique to SFB and VC, respectively. Alternatively, the discordant topologies between the trees of haplotypes and strains (Figs. 5 and 6, respectively) can be thought of as a gene-tree versus species-tree discrepancy related to the sorting of polymorphisms (Birky et al. 1983; Page and Holmes 1998). In conclusion, the same evolutionary forces that have shaped variability in nuclear loci have produced distinctly different signatures in the mtDNA genome. The mtDNA sequence analysed in this study has provided some diagnostic power in comparing strains SFB and VC of *Artemia*. A characteristic temporal cline in the frequency of the global haplotype should be further investigated before explanations based on neutrality can be safely excluded.

The identification of strains with different genetic compositions and reproductive profiles is expected to contribute considerably to the understanding of molecular ecology and aquaculture practices involving *Artemia*. The brine shrimp can be considered a model organism, offering numerous advantages for comprehensive, multidisciplinary studies.

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