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A general purpose genotype in an ancient asexual

Abstract Many parthenogenetic species are geographically more widely distributed than their sexual relatives. Their success has been partly attributed to the existence of general purpose genotypes (GPGs). *Darwinula stevensoni* is an ostracod species, which has persisted for >25 million years without sex, and is both ubiquitous and cosmopolitan. Here, we test the hypothesis that this ancient asexual species may possess a highly generalised (or general purpose) genotype. The ecological tolerance of *D. stevensoni* was compared with asexual populations of *Heterocypris incongruens*, a common cypridinid species with mixed reproduction, as well as with that of another ancient asexual darwinulid species with a limited geographic and ecological distribution, *Vestalenula molopoensis*. The unusually wide tolerance range for both salinity (0–30 g/l) and temperature (10°C, 20°C and 30°C) of the freshwater species *D. stevensoni*, supports the hypothesis that this ancient asexual has indeed developed a GPG. This coincides with its wide geographic and ecological distribution and might explain its persistence as an obligate asexual in its long-term evolution. The more restricted salinity tolerance of *V. molopoensis* (maximum at 12 g/l) shows that not all species of the ancient asexual family Darwinulidae have a GPG. *D. stevensoni* has a much broader tolerance than the asexuals of *H. incongruens*. We argue why a GPG is most likely to develop in long-term asexuals.

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Introduction

Whereas considerable controversy exists over the adaptive significance of sexual versus asexual reproduction (Maynard Smith 1978; Bell 1982; West et al. 1999) there is general agreement regarding the ecological and genetic difficulties that newly originated asexuals must face (Lynch 1984). The absence of mechanisms for rapid genetic change has earned asexuals the label of evolutionary dead ends (Maynard Smith 1978). Yet, many parthenogenetic species are geographically and ecologically widely distributed (reviewed in Bell 1982; Lynch 1984; Hughes 1989; Vrijenhoek 1998) suggesting that the limitation in genetic plasticity in parthenogenetic lineages can be compensated. The flourishing of asexual organisms in certain environments has been attributed to a variety of factors, including reproductive efficiency, faithful replication of general purpose genotypes (GPGs) and generation of specialised genotypes.

Asexuality facilitates colonisation of new habitats, because a single dispersing female or egg can establish a new population. This greater colonising ability may explain why asexuals are found more frequently at extreme latitudes and have a different distribution compared with their sexual relatives, a pattern known as geographic parthenogenesis (Vandel 1928). Several models attempt to explain this phenomenon. One of these holds that successful clones possess more broadly adapted ("general purpose") genotypes than sexual taxa (Baker 1965). Lynch (1984) argued that selection in a temporally varying environment will promote the evolution of clones with a GPG characterised by both broad tolerance ranges and low fitness variance across relevant physical, chemical and biotic gradients. The result is erosion of clonal diversity. A number of experimental tests of the existence of GPGs within asexual-sexual complexes have been conducted (Bierzychudek 1989; Weider 1993;

Parker and Niklasson 1995; Kenny 1996; Semlitsch et al. 1997; Browne and Wanigasekera 2000). The resulting data are contradictory, which is why no final conclusions has been drawn. Other data on GPG are only descriptive (Bell 1982; Lynch 1984). As the GPG hypothesis is thus far not corroborated, alternative explanations of geographic parthenogenesis have been proposed. One of these is the "frozen niche variation model" (FNV) of Vrijenhoek (1978, 1979) in which genetic variation, along with adaptations for a particular niche, is "frozen" in clonal lineages, arising through multiple independent origins (or polyphyletically) from sexual populations. Such lineages are generally considered ecological specialists. Originally, this model was developed to explain coexistence between sexual and asexual relatives. Parker (1979) however, argued that this model may be extended to predict that multiple colonisation by genetically diverse clones, instead of a GPG, enables asexuals to occupy wider geographic areas then sexual relatives.

The two models are mostly seen as antagonistic but in more general terms, the FNV and GPG models are not mutually exclusive. Clones are complex products of a "lucky draw" from the sexual gene pool and interclonal selection will remove bad combinations. It is therefore conceivable that different clones might have different properties, some more generalised others more specialised (Vrijenhoek and Pfeiler 1997; Tejedo et al. 2000). Most empirical studies indicate a high genetic diversity in those asexuals with mixed reproduction, which seems to be the result of the polyphyletic origin of these clones. As a consequence of this high clonal diversity, habitats are more likely to be occupied by differentially adapted clones rather than by a single highly generalised (or GPG) clone (Parker et al. 1977; Vrijenhoek 1979; Harshman and Futuyma 1985; Innes and Hebert 1988; Fox et al. 1996). This may explain why most studies on geographic parthenogenesis fail to demonstrate the existence of a genuine GPG in asexuals (Weider 1993; Parker and Niklasson 1995; Fox et al. 1996; Kenny 1996; Semlitsch et al. 1997; Browne and Wanigasekera 2000). Therefore, looking for a GPG in geographic parthenogenesis seems to be based on an erroneous assumption. Rather, a GPG should be sought in obligate asexuals where no sexual relatives occur. When a genotype originates in such an obligate asexual, it will be kept intact and gradually, over time, become either more specialised or more generalised through clonal selection. Therefore, we postulate that long-term asexuals are the most likely candidates to harbour a GPG.

Until now, the two putative ancient asexual groups are the bdelloid rotifers and the darwinulid ostracods (Judson and Normark 1996). Of these, the ostracods have a rich fossil record that shows that the family Darwinulidae has persisted without males for at least 100 million years (Martens 1998). *Darwinula stevensoni* has persisted asexually for 25 million years (Straub 1952) and is the oldest documented ancient asexual species. It shows almost no morphological (Rossetti and Martens 1998) and genetic (Schön et al. 1998) variability. It is furthermore common, ubiquitous and cosmopolitan (Griffiths and Butlin 1994). *D. stevensoni* is thus a promising candidate to test for the presence of a GPG.

A GPG is defined as having a wide tolerance, a low variability in response and a genotype capable of a wide geographic distribution. We thus test the tolerance and response of different populations of *D. stevensoni* to a wide range of salinities and temperatures. Stress tolerance across environments, measured as survival and mobility, is an indicator of fitness, which agrees well with the definition of GPG (Niklasson 1995).

D. stevensoni is unusual in the Darwinulidae, because no other species in this family has an equally wide geographical and ecological distribution. Twenty-two out of the 28 extant species are known from a few specimens and one locality only (Rossetti and Martens 1998). In our study we include *Vestalenula molopoensis*, a rare darwinulid from South Africa.

In order to test if obligate asexuality rather than mixed reproduction allows for highly generalised genotypes to develop, asexual clones from the ostracod *Heterocypris incongruens* were also tested. This species belongs to the common family Cyprididae (Martens 1998), is also cosmopolitan and ubiquitous, but has a mixed reproductive strategy with geographical parthenogenesis. It shows a high clonal variability (Rossi et al. 1998), and due to (inter- and intraspecific) hybridisation, at least some of its clones could have a polyphyletic origin. This study is a novel approach to the problem and compares different types of asexual lineages (obligate versus mixed), not asexuals versus sexuals.

Materials and methods

Source of ostracods

D. stevensoni was collected from three different European sites. The populations from Belgium (DsB) and Ireland (DsI) are both derived from lakes. The Belgian site is more saline than the Irish lake (EC=3,440 and 200 µS/cm respectively). The French population (DsF) lives interstitially near a warm water spring (EC=1,600 µS/cm). *V. molopoensis*, was collected from the outflow of a dolomitic spring in South Africa (EC=425 µS/cm); the species is known from this area only (Rossetti and Martens 1998). Asexual populations of *H. incongruens* were collected from two localities in Belgium, a horse trough (Hi1) and a temporary pond (Hi2) (EC=649 and 547 µS/cm respectively). Because *D. stevensoni* has a life cycle of >3 years and a low fecundity [maximum 20 eggs per generation (Ranta 1979)] monoclonal laboratory cultures could not be obtained. To allow for comparison, organisms from the other species were also derived directly from the field.

Genetic variability

D. stevensoni was sampled from sites where monoclonal populations are known to occur (Schön et al. 1998). Genetic variability of the *H. incongruens* populations was studied with cellulose acetate electrophoresis to check for monoclonality (Hebert and Beaton 1993). Individuals were crushed separately and the genotype of 70 individuals from each population was screened for six enzyme loci: 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase 1 and 2 (IDH1, IDH2), phosphoglucose isomerase (PGI), mannose phosphate isomerase and phosphoglucomutase.

Experimental design

Water used for the experiments was collected in the same localities (see above) and every population was tested in medium from its source habitat. Chemical composition and electrical conductivity were determined using a DREL/5 spectrophotometer (HACH) and an LF 325 conductivity meter. Total dissolved solids were calculated, for comparative purposes, with the formula of Williams (Williams 1966). After filtering (5 µm), experimental salinities were obtained by adding pure salts (NaCl, KCl, $NaHCO_3$, $CaCl_2$ and $MgSO_4$). The original relative composition of the habitat waters from DsF and DsI could not be maintained at higher salinities because these waters were carbonated (also dominated by SO_4^{2-}). The formula of Williams is only applicable for NaCl lakes, like for DsB. The composition of this water was therefore used to obtain the salinities from the other sites, which could give a slight advantage to the individuals of DsB.

Tested individuals were acclimatised in their filtered habitat water for 4 weeks prior to testing (17°C, photoperiod 12:12 h light:dark). In each experiment, organisms were subjected to combinations of eight salinities (0, 4, 8, 12, 16, 20, 25 and 30 g/l) and three temperatures (10, 20 and 30°C), resulting in 24 treatments. Each treatment involved five individuals and was conducted 3 times with different individuals. For each set of replicates, a control of ten organisms was used. Each tolerance test thus used 390 individuals from each population. Individual ostracods were picked out randomly from the acclimatisation stock and transferred separately to 25-ml bottles filled with 15 ml of the desired solution. Each bottle was closed with a lid to prevent evaporation. All bottles were kept in boxes maintained at constant temperatures, through a bain-marie system, and at a photoperiod of 12:12 h light:dark.

Measures of fitness

Survival and mobility were checked every 4 h on the first day and then every 12 h until 72 h had elapsed. After the exposure, nonmobile organisms were transferred to acclimatisation conditions (see above) and survival was checked. Because most ostracods were closed and immobile during the exposure, survival at different time intervals could not always be checked. Analyses therefore use survival for each individual at the end of the experiment, expressed as dead (0) or alive (1). Mobility was checked at each time interval. Again because of the long generation time of *D. stevensoni*, life history parameters could not be used as measures of fitness.

Statistical analyses

Data from the same population are statistically correlated and not independent. In addition, animals from the same replicate may exhibit more correlation with each other than with animals from the other replicates. In this case, it is inappropriate to analyse the data using a standard linear model. A way to model the correlation is through the use of a replicate/population random effect in a mixed model regression approach (Verbeke and Molenberghs 1997). Defining replicate/population as a random effect sets up a common correlation among all observations having the same level of replicate/population. We analysed differences in survival among populations and species using a mixed model logistic regression with binomial errors and logit transformation (Littell et al. 1996; Neter et al. 1996). To estimate time to immobility, a Weibull survival model was fitted (Klein and Moeschburger 1997). Because time registrations are interval censored, a complementary log-log model for continuous-time processes was used (Allison 1995).

For the comparison of populations within species, we added replicate as a random effect, to neutralise possible pseudoreplication effects due to correlation among replicate members. For the comparison among species, we added population as a random effect to test species' effects against population level variation. In each case, the intercepts and slopes for salinity and temperature were included in the random statement to account for replicate/population-specific variation. Salinity, temperature and population or species were tested as fixed effects. To test the significance of effects in mixed models, error terms must be constructed that contain all the same sources of random variation except for the variation of the respective effect of interest. In this case, the *df* were approximated by the Satterthwaite formula (Satterthwaite 1941). The need of the random effects in the model was tested with the likelihood ratio test and the use of the Akaike information criterion (Verbeke and Molenberghs 1997). The models were fitted with the GLIMMIX macro in SAS 8.02 (Littell et al. 1996). Variance components were estimated by restricted maximum likelihood.

Results

Ecological tolerance of *D. stevensoni*

There were no significant random effects (all $P>0.05$) in the mixed model regressions for *D. stevensoni*, revealing that there were no replicate dependencies. There were significant effects of salinity and temperature on survival of *D. stevensoni*; no significant salinity-by-temperature interactions were observed (Table 1). Survival was lower at higher temperatures and at higher salinities but even then, this freshwater species showed a high survival rate in salinities approaching that of seawater (30 g/l). DsB showed a significantly broader tolerance towards all treatment combinations than the other two populations, DsF and DsI (Fig. 1, Table 1). The survival response

Table 1 Effects on survival of salinity, temperature, population and their interactions for each species, analysed by the mixed model logistic regression. Non-significant factors ($P>0.05$) are not given. For *Darwinula stevensoni* (Ds), population-specific contrasts were tested and the effects of all factors are presented. *DsB* Belgian Ds population, *DsF* French Ds population, *DsI* Irish Ds population

Species	Source	*df*	*F*	*P*
Darwinula stevensoni	Temperature	1, 1065	11.08	0.0009
	Salinity	1, 1065	61.83	0.0001
	Population	2, 1065	9.81	0.0001
Heterocypris incongruens	Temperature	1, 714	69.48	0.0001
	Salinity	1, 714	48.16	0.0001
	Temperature×salinity	1, 714	17.16	0.0001
Vestalenula molopoensis	Salinity	1, 358	38.46	0.0001
Specific population differences				
Darwinula stevensoni	DsB-DsF	1, 1065	16.38	0.0001
	DsB-DsI	1, 1065	16.15	0.0001
	DsF-DsI	1, 1065	0.00	0.9580

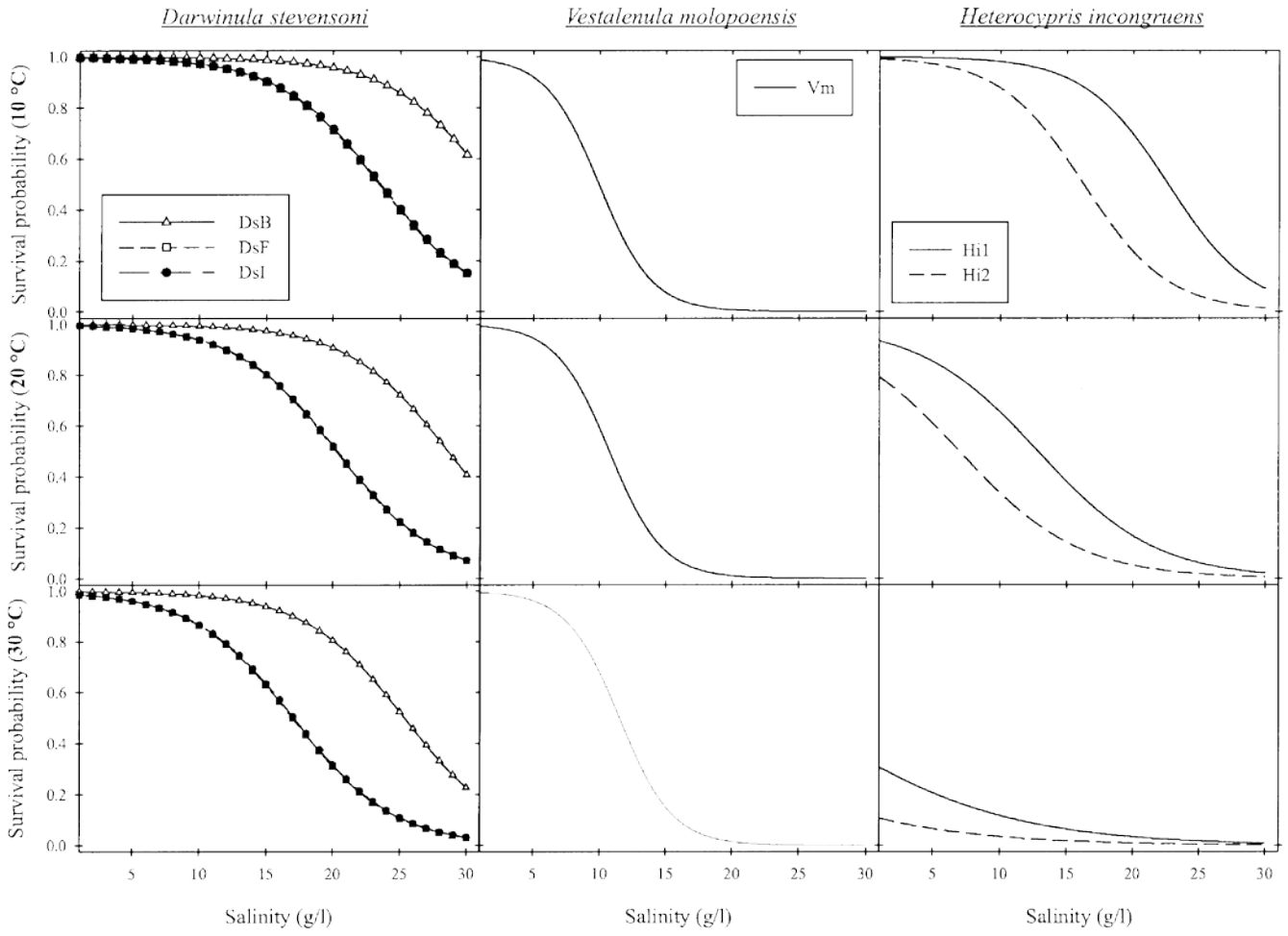

Fig. 1 Survival as a function of salinity and temperature as estimated by GLIMMIX. The graphs show the output of the model based on the observed data. Note the nearly identical response of the populations of *Darwinula stevensoni* (Ds) taken from near a warm water spring in France (*DsF*) and an Irish (*DsI*) lake. *DsB* Belgian Ds population from a lake, *Hi1* Belgian population of asexual *Heterocypris incongruens* (Hi) from a horse trough, *Hi2* Belgian population of asexual Hi from a temporary pond, *Vm Vestalenula molopoensis*

curves of the latter two populations are nearly identical. A significant effect of the three-way-interaction salinity×temperature×population on mobility of *D. stevensoni* was observed: effects of different temperature and salinity concentrations were dependent on "population" (Table 2). All three tested populations had a tendency towards longer mobility at the highest temperature (30°C): DsB being the most mobile, DsI the least.

Ecological tolerance of *V. molopoensis*

No significant random effects (all $P>0.05$) were revealed in the mixed model regressions. Salinity had a strong effect on survival of *V. molopoensis* (Table 1). Neither temperature nor salinity-by-temperature interactions had a significant effect. Individuals of this species died at a salinity of 12 g/l and more, independent of temperature (Fig. 1). The Weibull survival model showed a significant effect of the two-way interaction salinity×temperature on the mobility of *V. molopoensis* (Table 2). At higher temperature (30°C) a tendency towards higher mobility was detected.

Clonal variability and ecological tolerance of *H. incongruens*

Four different multilocus genotypes (using PGI, IDH1, IDH2) were identified from population Hi2 (clonal frequencies were 55:17:14:13%). The other tested allozyme markers showed no variability. Population Hi1 was monoclonal for the studied allozyme markers and showed the same allozyme pattern as the most common clone of population Hi2. There were no significant random effects (all $P>0.05$) in the mixed model regressions and no significant difference in survival between Hi1 and Hi2. Significant effects of salinity, temperature and salinity-by-temperature interactions on survival were revealed for both populations. The effect of the different salinity concentrations depended on temperature, with no individuals surviving at 30°C (Fig. 1). At 10°C, individuals of both populations could tolerate salinities of up to

Table 2 Effects on mobility of salinity, temperature, population and their interactions for each species, analysed by the Weibull survival model. Non-significant factors (P>0.05) are not presented

Species	Source	*df*	*F*	*P*
D. stevensoni	Salinity	1, 4490.90	41.37	0.0001
	Population	2, 659.06	12.86	0.0001
	Temperature×population	2, 4490.04	3.26	0.0386
	Salinity×population	2, 4490.70	5.47	0.0042
	Temperature×salinity×population	2, 4488.41	4.20	0.0151
H. incongruens	Temperature	1, 3680.74	153.60	0.0001
	Salinity	1, 3680.36	189.80	0.0001
	Temperature×salinity	1, 3685.92	86.16	0.0001
	Population	1, 10.54	8.14	0.0163
	Salinity×population	1, 3683.60	10.22	0.0014
V. molopoensis	Salinity	1, 1496	50.21	0.0001
	Temperature×salinity	1, 1496	9.95	0.0016

16 g/l, rarely up to 20 g/l. The Weibull survival model demonstrated significant effects of temperature, salinity, salinity-by-temperature interaction and salinity-by-population interaction on mobility (Table 2). At temperatures of 10 and 20°C and salinities of up to 16 g/l, all individuals remained mobile. At 30°C, mobility was interrupted at maximum tolerance.

Comparison of the different species

There were no significant random effects (all P>0.05) in the mixed model logistic regression for survival. This was already indicated by the species-specific analyses where no population differences in response to temperature and salinity were observed. There were significant effects on survival of temperature ($F_{1,2136.8}$=11.72, P=0.0006), salinity ($F_{1,2136.05}$=33.09, P=0.0001) and the two-way interaction, temperature×species ($F_{2,2136.41}$=9.33, P=0.0001) and a tendency for a salinity×species interaction ($F_{2,2115.90}$=2.69, P=0.0683). The effect of different temperatures thus depended on species, with *V. molopoensis* being the least and *H. incongruens* being the most sensitive (Fig. 2). *D. stevensoni* showed a tendency towards higher mortality at higher temperature. The effect of the various salinities also depended on species, with *V. molopoensis* being the most sensitive (Fig. 2). *D. stevensoni* had a clearly broader tolerance for all treatment combinations than the other tested species (Fig. 2).

As expected from the species-specific analyses, there was a significant random effect for temperature and salinity in the mixed model for mobility (P<0.001). Mobility was significantly affected by the three-way interaction temperature×salinity×species ($F_{1,4577}$=12.62; P<0.0001); the effects of temperature and salinity thus depend on species. A different response was observed for *H. incongruens* relative to the darwinulid ostracods at lower temperatures (Fig. 3), with the darwinulids being almost immobile. At the highest temperature this effect disappeared.

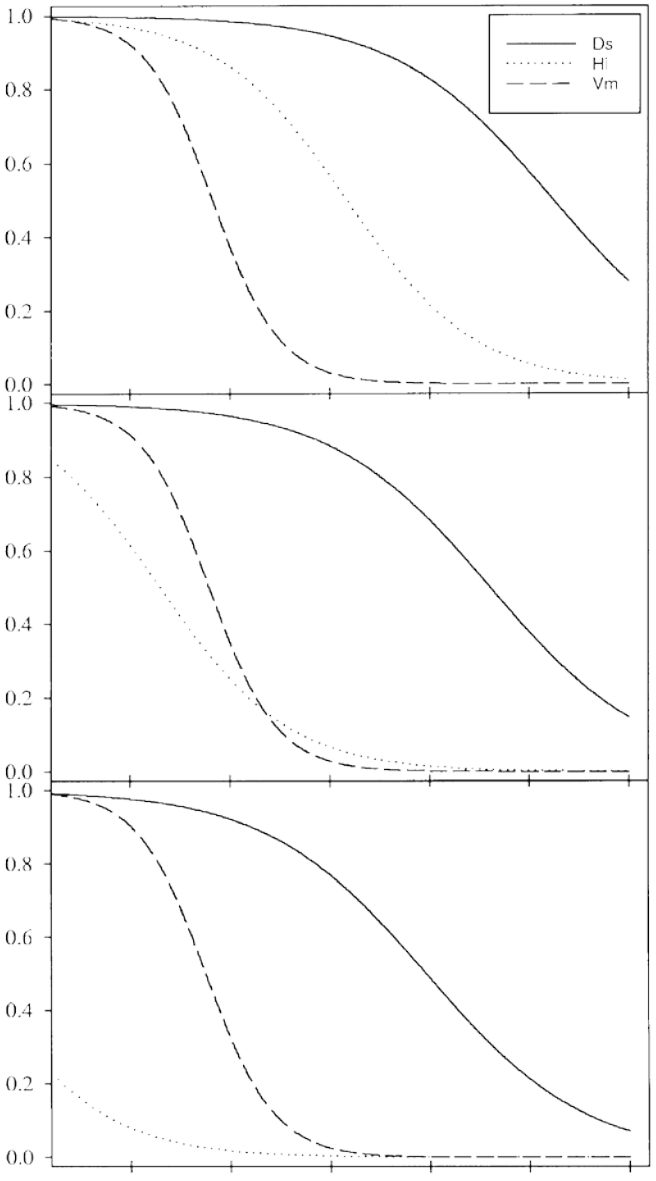

Fig. 2 Survival as a function of salinity and temperature for the three species, as estimated by GLIMMIX. The graph is the output of the model based on the observed data. For abbreviations, see Fig. 1

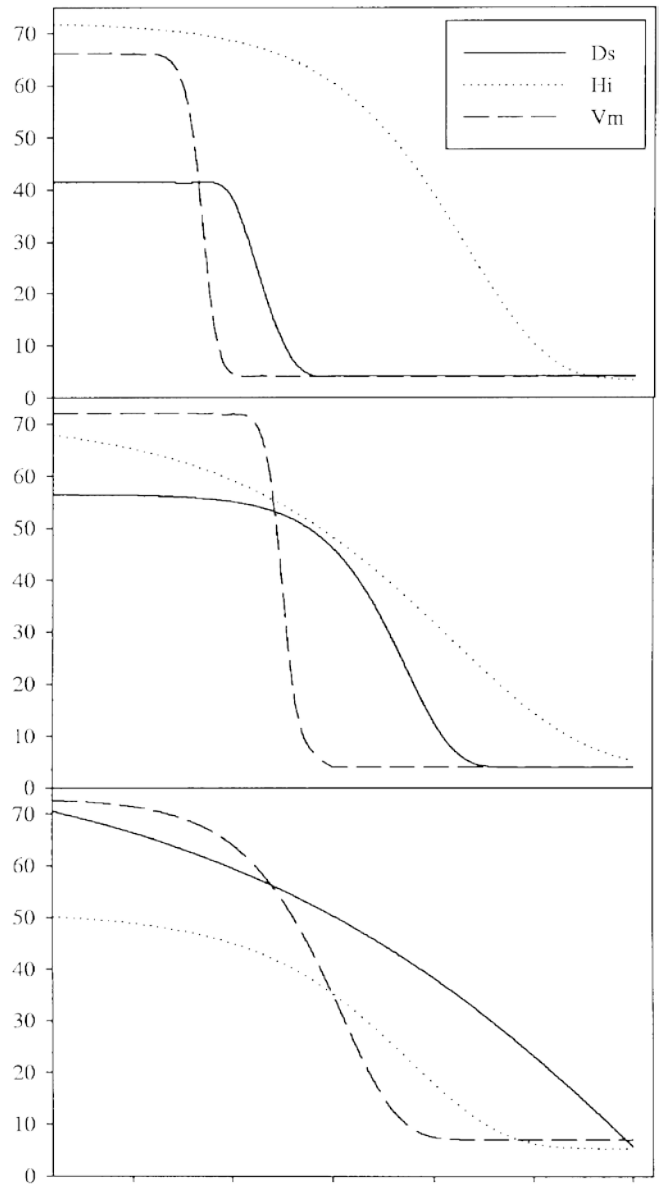

Fig. 3 Time as a function of salinity and temperature for the three species together as estimated by the Weibull survival model. For abbreviations, see Fig. 1

Discussion

A GPG in *D. stevensoni*

The results presented above strongly support the notion that *D. stevensoni*, a species that has been asexual for a long evolutionary time (25 million years), has a more generalised genotype than the asexuals from *H. incongruens*, a species with mixed reproduction.

This study demonstrates that *D. stevensoni* cannot only withstand salinities approaching 30 g/l, but also can survive in distilled water independent of the temperature. Most freshwater organisms are not able to sustain salinities above 10 g/l, rarely up to 20 g/l (Beadle 1969). *D. stevensoni* is therefore an exceptional freshwater species with regard to its salinity tolerance. The two populations of *H. incongruens* tested, were more sensitive to thermal and salinity stress than *D. stevensoni*: all individuals died at 30°C, independent of salinity and of the mono- or polyclonality of the source populations. *V. molopoensis* was quite tolerant to temperature, but not to salinity changes. This illustrates how different types of selection may lead to different types of tolerance, with *D. stevensoni* being tolerant to both environmental variables.

In the search for GPGs in natural populations of parthenogens the definition is usually based on the ecological and geographical ranges the genotype is able to occupy and the low variability in response to environmental conditions among genotypes and populations (see above). It was already known that *D. stevensoni* has a wide geographical and ecological distribution (Rossetti and Martens 1998). In this study we have demonstrated that the strategy underlying this wide distribution is a genotype providing the organism with a phenotype capable of wide environmental tolerance. Both results corroborate the hypothesis that *D. stevensoni* has a GPG. The low variability in response among populations was also tested and partly corroborated in this study. Populations DsF and DsI, isolated from different habitat types (see above), indeed had nearly identical responses to the different treatments. The population from Belgium (DsB) however, shows a significantly higher tolerance. This difference may be caused by genetic (nature) or environmental (nurture) factors or by a genotype-environment interaction (Maynard Smith 1998). We cannot exclude the possibility that the difference has a genetic component, although it has been shown that the populations DsB and DsF are both monoclonal with identical genotypes for the screened genes and allozymes (Rossi et al. 1998; Schön et al. 1998). The discrepancy in response can be caused by differences between the environments in which these populations developed, given that the animals were isolated directly from their habitat. The habitat inhabited by DsB shows both a higher mean salinity and stronger salinity fluctuations than either DsF and DsI. In addition, the experimental design used the ionic composition of the habitat water from DsB to prepare the different salinity solutions for testing of the other populations. Phenotypes of DsB individuals were thus better acclimatised to the test solutions. If the wider tolerance of DsB individuals represents a phenotypic adaptation, then our results are still in accordance with the GPG hypothesis.

Tolerance of *V. molopoensis*

The present results also demonstrate that at least one other species of the ancient asexual family Darwinulidae is not a generalist. *V. molopoensis* has a salinity tolerance typical of freshwater organisms (see above) with a maximum of 12 g/l. This agrees with the restricted geographic distribution of *V. molopoensis* (Rossetti and Martens

1998) and may indicate ecological specialisation. The geographical distribution and ecological preferences of most of the other 28 species within the family Darwinulidae are more similar to those of *V. molopoensis* than to *D. stevensoni* (Rossetti and Martens 1998).

From the existing data, it is difficult to draw final conclusions about the ecological tolerance of the whole family but it seems that the GPG of *D. stevensoni* constitutes more the exception than the rule.

Strategies for long-term survival in asexuals

H. incongruens is a cosmopolitan species but does not have a GPG. This species seems to have developed an evolutionary strategy different from a GPG: asexuals are specialised and adapted to specific habitat characteristics.

Parker et al. (1977) stated that over evolutionary periods of time, clonal selection would sort out the most tolerant genotypes of the originally coexisting assemblage (see also White 1973). However, in a diverse set of polyphyletic clones generated from a mixed sexual-asexual cluster and exhibiting differential survival, selection may equally likely result in adaptation to specific habitat characteristics (Hughes 1989). Generalised clones may appear but seem not to evolve to a GPG and often will be outcompeted by the local specialised clones (Roughgarden 1972). Thus, the widespread occurrence of asexuals, within an asexual-sexual complex, seems more likely to be supported by the general performance superiority of clonal mixtures (see FNV model) instead of a single GPG clone.

In an old and fully asexual lineage, such as the ancient asexual family Darwinulidae, it is conceivable that initial tendencies for ecological generalisation or specialisation may be accentuated during the course of time. Generalist and specialist species are therefore expected and also found: beside the generalist *D. stevensoni*, more specialised species like *V. molopoensis* have apparently evolved in the Darwinulidae.

It is our hypothesis that both generalists and specialists have originated in the Darwinulidae as lucky draws, possibly from sexual ancestors (>200 million years ago) or through mutations from asexual ancestors. Through time, selection has resulted in the development of a true GPG in the generalist lineage. This is because selection in fully asexual lineages acts at the level of the entire genotype (for review see Templeton 1982) and a truly generalised clone with a wide ecological tolerance may thus evolve and displace inferior genotypes. As there are no sexual relatives, there is no hybridisation and this genotype can be transmitted intact from generation to generation. The specialised lineages (like *V. molopoensis*) will probably be short-lived compared to the extreme GPG species and become extinct without attaining wide distribution. It might be that these specialised lineages are recent offshoots from a putative core lineage with a GPG.

In order to persist over long evolutionary times, such a GPG must be protected from mutational meltdown (Kondrashov 1993) and Muller's ratchet (Muller 1964). Efficient DNA repair, in the strict sense of correctly repairing mutated bases, has been suggested as a plausible mechanism (Schön and Martens 1998; Judson and Normark 2000).

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