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Clonal diversity in the ancient asexual ostracod *Darwinula stevensoni* assessed by RAPD-PCR

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As Darwinulidae (Ostracoda) are considered to be ancient asexuals with a wide geographical and ecological distribution, they are expected to have accumulated mutations during a long timeframe. However, previous studies on genetic variability suggested a low genetic divergence within the darwinulid species *Darwinula stevensoni*.

Here, overall genotopic diversity of *D. stevensoni* is estimated with the Random Amplified Polymorphic DNA (RAPD) technique. Using six primers revealing 47 consistently scorable polymorphic loci, substantial clonal diversity within this species is detected. Five of the seven surveyed populations are multiclonal. Moreover, the seven populations have a different clonal composition with almost all of the observed clonal genotypes being restricted to single populations, indicating the absence of a single widespread 'clone'.

The observed clonal diversity seems to refute the existence of a widespread general purpose genotype for *D. stevensoni*. However, in light of previously detected uniformity at functional loci, we reconsider the definition of a GPG. We suggest that it need not imply a genome-wide fixed genotype, but rather consists of a set of ecologically relevant genes.

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Introduction

The Ostracoda are a unique animal group in which an extensive range of reproductive strategies occurs together with a high incidence of transitions from sexual to asexual reproduction (Martens, 1998). Allozyme studies of mother–daughter pedigrees have provided some evidence that the latter occurs apomictically (see Chaplin, 1992; Havel and Hebert, 1993; Rossi *et al*, 1993).

The classical notion that asexual organisms have low levels of genetic variation turns out to be the exception rather than the rule, and depends on clonal ancestry (Parker, 1979), the amount of recurrent hybridisation with sexual relatives (Lynch *et al*, 1989), automictic events (Little and Hebert, 1994) and the frequency of mixis in the life cycle (Hughes, 1989; Carvalho, 1994). Most asexual ostracod populations hitherto studied belong to species with mixed reproduction and show high clonal diversities (reviewed in Havel and Hebert, 1993; Rossi *et al*, 1998). Some of this clonal variation has undoubtedly arisen from mutations, but the high levels are mainly the result of repeated clonal origins from bisexual conspecifics and/or occasional hybridisation between asexual females and males (Chaplin *et al*, 1994; Little and Hebert, 1994; Schön *et al*, 2000 and references therein). The joint occurrence of sexual and asexual taxa in some genera suggests that asexual ostracods evolved recently (Chaplin *et al*, 1994). However, the family Darwinulidae seems to provide a striking exception to this latter pattern. Darwinulids are considered to be ancient asexuals because their fossil record shows a complete absence of males since the Triassic, that is, during the last 200 million years (Martens *et al*, 2003). The most common species of this family, *Darwinula stevensoni*, is assumed to have reproduced apomictically (Butlin *et al*, 1998) for at least 25 myr (Straub, 1952). As this species has no sexual relatives, interbreeding and recurrent origins of clones could not have generated clonal diversity, so that new mutations are probably the only source of variation. Hence, when this process operates over long timeframes, genetic differences are expected to continuously accumulate between clonal lineages (Butlin *et al*, 1998) and, since recombination is absent, also between alleles within lineages (Mark Welch and Meselson, 2000). In contrast with these expectations, little genetic variation has thus far been found within *D. stevensoni* by allozyme screening and DNA sequencing (see Havel and Hebert, 1993; Rossi *et al*, 1998; Schön *et al*, 1998; Schön and Martens, 2003). This apparently low genetic variability is intriguing considering the wide geographical and ecological distribution of *D. stevensoni* (Rossetti and Martens, 1998).

Generally, the widespread distribution of asexual lineages is explained either through dispersal of a single clonal lineage with wide ecological tolerance (ie general purpose genotypes or GPG) or by the presence of many locally adapted clones. Circumstantial evidence for the wide ecological tolerance of asexual species was

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provided by the broad geographical distribution of a number of asexuals compared to their sexual relatives (Lynch, 1984; Parker and Niklasson, 1998 and references therein). Vrijenhoek (1998) however asserted that molecular markers should be used in order to investigate whether a widespread asexual taxon is composed of a single plastic clonal lineage (a GPG) or of several ecologically specialised clones. Understanding the geographic distribution of asexuals can thus be partly achieved through a combined analysis of their population genetics and environmental tolerance (Jacobsen *et al*, 1996; Vrijenhoek, 1998).

Recently, Van Doninck *et al* (2002) showed that *D. stevensoni* had a higher tolerance for salinity and temperature than other asexual ostracods, while Rossi *et al* (2002) reported a high hypoxia tolerance for *D. stevensoni*. These results suggest that *D. stevensoni* shows the ecological characteristics of a GPG. However, although current genetic data from *D. stevensoni*, using several genetic markers, seem to suggest that these ecological characteristic are associated with a low genetic variability (Rossi *et al*, 1998; Schön *et al*, 1998; Schön and Martens, 2003), overall genotypic diversity in this species remains unknown and needs investigation.

Therefore, the aim of the present study is to estimate clonal diversity in *D. stevensoni* from different geographic populations using genome-wide genetic markers. In this way, this study investigates (1) the degree of clonal similarity within and between *D. stevensoni* populations, (2) to what extent *D. stevensoni* populations are dominated by one or a few widespread clonal genotypes and (3) whether the *D. stevensoni* populations of the ecological study of Van Doninck *et al* (2002) are monoclonal and genetically identical. To achieve these goals, the RAPD-PCR priming technique was applied (Williams *et al*, 1990).

Materials and methods

Sample collection

Seven populations of *D. stevensoni* were surveyed by means of RAPD-PCR (Table 1), three of which BH, FC and IB were also included in previous ecological tests and were then labelled DsB, DsF and DsI, respectively (Van Doninck *et al*, 2002). After field collection, specimens were maintained alive in the laboratory in plastic sampling bottles with sediment and water of the sampling location. Bottles were continuously oxygenated and distilled water was added to compensate for evaporation. Prior to DNA extraction, individuals were starved for at least 48 h in distilled water, transferred individually to 1.5 ml eppendorf tubes and stored at -80°C.

DNA extraction

Four different methods to extract DNA from individual ostracods were compared, viz: (1) Chelex extraction according to Schön *et al* (1998), (2) the CTAB protocol used for mites (Van Opijnen and Breeuwer, 1999), (3) the Gene Releaser protocol of Schizas *et al* (1997) (for harpacticoid copepods) and Oakley (pers. comm.) (for ostracods), and (4) the protocol implemented by QIAamp DNA Mini kit (Qiagen) for extractions of tissues, using 50 µl DNA elution buffer instead of 200 µl in the last two steps. Extracted DNA was stored at -20°C.

Optimisation of RAPD procedure

To minimise experimental errors causing artifactual RAPD bands, the amplification protocols were optimised and standardised so that bands were consistent across replicates (see Black IV, 1993; Guirao *et al*, 1995). To this end, a series of optimisation experiments were conducted in which concentrations of DNA template, primers, nucleotides, $MgCl_2$ and *Taq* polymerase were varied to determine the conditions yielding the most intense banding patterns. The optimised reaction conditions were as follows: total reaction volumes of 25 µl were used with final concentrations of 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 100 µM dNTP (Promega), 0.2 µM primer, 0.5 U *Taq* polymerase (Pharmacia) and 2 µl DNA from a stock solution of 100 µl. Amplifications were conducted in a Biometra (Tgradient) Thermal Cycler (Westburg) with the following settings: 5 min denaturation at 95°C, followed by 40 cycles of 1 min at 95°C, annealing at 37°C for 1 min and elongation at 72°C for 2 min. An additional 10 min elongation at 72°C followed the last cycle. Amplification products were electrophoresed on 6% polyacrylamide gels at 180 V for 5 h in 1 × TBE buffer (1 M Tris, 0.83 M Boric Acid, 10 mM EDTA) together with a 100 bp ladder (Pharmacia). After electrophoresis, gels were silver stained. Agarose gels, stained with ethidium bromide, were also used for electrophoresis during optimisation.

A prescreening with 52 primers (six from Pharmacia and 46 from Operon primer kits OPA, OPB and OPE) was also conducted, using one to three individuals of each population. Only those primers that yielded positive amplifications and revealed well visible RAPD fragments for all populations were used for further study. Next, those selected primers were tested in triplicate to investigate their reproducibility; that is using

Table 1 Populations of *D. stevensoni* used in RAPD analysis

Code	*Site*	*n*	N_c	N_c/n	G_0	G_0/N_c	*h*
BM	Grand Mellaerts, Belgium	20	5	0.25	3.52	0.70	0.086
BH	Hollandersgatkreek, Belgium	21	1	0.048	1	1	0
BT	Tenreuken, Belgium	25	8	0.32	3.93	0.491	0.123
FC	Clue de la Fou, France	17	1	0.059	1	1	0
IB	Lough Ballyquirke, Ireland	21	2	0.095	1.6	0.8	0.005
IL	Lough Lickeen, Ireland	12	2	0.167	1.8	0.9	0.017
SC	Caveta, Spain	25	14	0.56	7.53	0.538	0.119

The sample size per population (*n*), number of clones (N_c), three measures of genotypic diversity (N_c/n, G_0 and G_0/N_c) and Nei's (1973) gene diversity (*h*) are given for each tested population.

the same primers, the same DNA stock (from individuals of the seven populations) and the same batch of reagents, three independent PCRs were conducted, electrophoresed on different gels and compared to investigate whether that primer revealed polymorphic, reproducible bands for each tested population. Six primers, showing 100% reproducibility of distinct bands, were retained and used to analyse variation in *D. stevensoni*, viz OPA-2 (TGCCGAGCTG), OPA-8 (GTGACGTAGG), OPA-10 (GT GATCGCAG), OPB-7 (GGTGACGCAG), OPB-10 (CTGC TGGGAC) and OPE-4 (GTGACATGCC).

In order to examine whether epibionts on carapaces of specimens could affect the RAPD banding patterns, DNA was extracted only from the bodies of at least three individuals from each population and analysed in the optimisation phase.

Study of genetic variation

For the experiment, at least 20 individuals of each *D. stevensoni* population were analysed, except for populations IL (12) and FC (17) (Table 1). All RAPD-PCR reactions were repeated twice: using the same primers and individuals, the replicate PCRs were conducted independently on different days. Samples from previous amplifications were included in all gel runs to ensure comparability of band profiles. Furthermore, for each primer, all different genotypes detected in the seven populations were re-evaluated by running them simultaneously on single gels. Only polymorphic bands that were distinct, intense and completely reproducible were scored (presence/absence: p/a) (Figure 1). Multilocus genotypes were then defined by combining p/a data for the scored polymorphic RAPD bands. Thus, genotypes differing by a single band from each other were considered to be different clonal genotypes. Negative controls were included in each PCR reaction to ensure that all RAPD bands resulted from template DNA only.

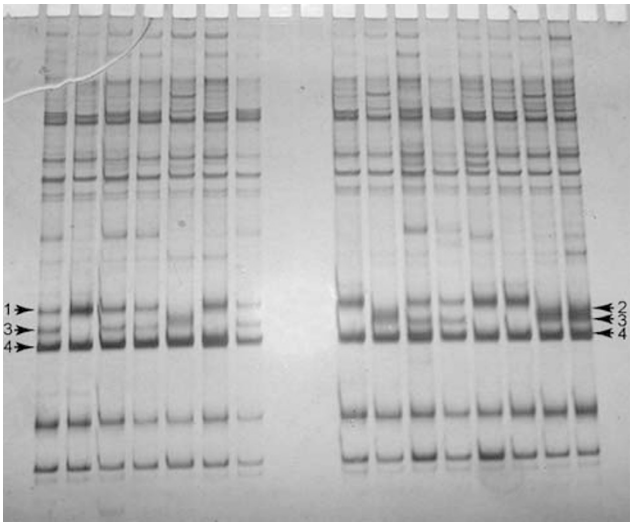

Figure 1 RAPD profiles of *D. stevensoni* from Grand Mellaerts, Belgium (BM) amplified with primer OPA-2. The lanes indicated by an arrow and numbered were the distinct, polymorphic bands that were scored for this specific population and primer; the other bands present on the gel were not scored because they were not 100% reproducible. Note that three different genotypes can be inferred from the four scored bands (presence/absence data).

Two components of genetic variation were evaluated. The first component was Nei's (1973) gene diversity h as estimated with the computer program POPGENE v. 1.20 (Yeh and Boyle, 1997). The second component was the clonal diversity, for which three indices were calculated: (1) the proportion of distinguishable clones N_c/n with N_c being the number of clonal genotypes and n the population size; when all individuals have a unique genotype N_c/n equals 1, whereas N_c/n approaches 0 when there are few clonal genotypes (Chen *et al*, 2002); (2) the observed genotypic diversity $G_0 = 1/(\sum_i^k g_i^2)$ where g_i is the frequency of the ith genotype (or clone) and k the total number of genotypes (Stoddart, 1983); G_0 varies from a minimum of 1, when there is a single genotype, to a maximum of k, when genotypes are evenly distributed; (3) the genotypic evenness (ie the number of individuals per clonal genotype), G_0/N_c, has a maximum value of 1 when all individuals are distributed evenly among the clones and will approach 0 if a single clone dominates the population (Coffroth and Lasker, 1998). For comparison, these indices were also calculated from asexual populations of other species for which sufficient multilocus data from allozyme, RAPD or microsatellite analyses were available (the complete review with the calculated population parameters is available from KVD upon request).

Band similarities between multilocus genotypes were inferred using the Nei and Li (1979) coefficient (S) calculated by the NT-SYS v. 1.80 software (Rohlf, 1993), in which this coefficient is referred to as 'Dice coefficient'. S-values were subjected to UPGMA clustering using the same program. The stability of the UPGMA topology was assessed via bootstrapping over 1000 replicates using the program WinBoot (Yap and Nelson 1996).

Results

Reproducibility

Optimal DNA extractions were achieved with the QIAamp DNA Mini kit, by the criterion that sharper RAPD bands were obtained with this method. In this study, polyacrylamide gels were used instead of agarose gels because more bands per primer were visible and bands were intenser, resulting in a higher resolution and a more accurate scoring (see also Fukatsu and Ishikawa, 1994).

As only those primers revealing a clear, intense banding pattern were selected during the optimisation and a conservative approach was followed in scoring only clear, totally reproducible bands, we encountered no difficulties in obtaining reproducible RAPD profiles. One primer revealed between four and 12 scorable loci (or bands) for all seven populations. Additional bands present on the gel were not scored because they were not distinct or reproducible (see Figure 1). Moreover, the scoring of bands was made directly from the gel. As a result, scored polymorphic bands were identical between replicates of the same individuals (eg Figure 2).

Clonal population structure

The six RAPD primers yielded 47 consistently scorable, polymorphic bands (or loci), which together defined 33 multilocus genotypes (here further referred to as 'clones') in 141 individuals of seven *D. stevensoni* populations.

Populations BH and FC showed no genotypic variation ($N_c = 1$). The other populations consisted of several clones (N_c ranged from 2 to 14) with most clones (14) being detected in the Spanish population SC (Table 1). Population IL was included in these calculations of genetic variation because it clearly represented another clone. Yet in the calculations of Nei and Li's (1979) coefficient (*S*) and their UPGMA clustering, IL was excluded because of incomplete data. This latter analysis showed that clones clustered by geographic region (Belgium, France, Ireland and Spain), while no genotype was found in more than one region (Figure 3). In Belgium, where more than one population was sampled, clones largely cluster by population, but this patterning is not fully bootstrap supported. Moreover, one clone from BT clustered with population BM, though with low bootstrap support (Figure 3).

Within each of the analysed populations, clones differed from each other only by single RAPD bands while most clones from different populations differed by at least 15 bands. In addition, none of the clones was present in more than one population.

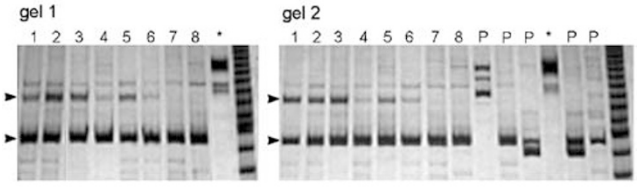

Figure 2 RAPD profiles of *D. stevensoni* from Tenreuken, Belgium (BT), amplified with primer OPA-8. The numbered lanes of gel 1 and gel 2 are replicates from subsequent PCR, each representing an individual. The lane labelled with * is a reference, included in the different gel runs. Lanes labelled with the letter P represent RAPD profiles from other tested populations amplified with OPA-8. The distinct, polymorphic bands scored only for population BT are indicated with an arrow.

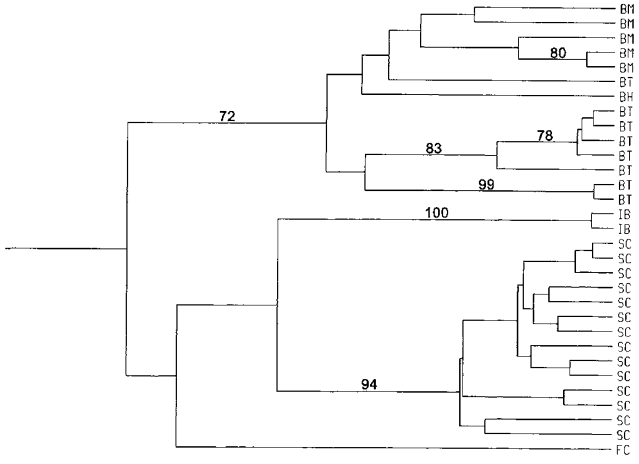

Figure 3 UPGMA dendrogram showing the relationships among *D. stevensoni* populations. Numbers represent bootstrap values (1000 replicates).

Genotypic diversity

Taken over the seven populations studied here, the observed genotypic diversity G_0 for *D. stevensoni* varied between 1 and 7.53, the proportion of distinguishable clones N_c/n ranged from 0.048 to 0.56 (Tables 1 and 2).

The populations with the lowest G_0 and N_c/n are BH and FC, in which the individuals belong to the same clone. Within each population from Ireland (IB and IL) two clones were found, differing from each other by a single band. Populations BM, BT and SC contained five, eight and 14 clones respectively (Table 1). Evenness, G_0/N_c, also varied among the seven populations, ranging between 0.491 and 1.000 (Table 1). For the two populations, BT and SC, the numbers of individuals per clone was not evenly distributed ($G_0/N_c < 0.55$, Table 1). Among the BT population, two clones were found with eight and nine individuals and five clones with only a single individual. For the Spanish population (SC) we found one clone with seven individuals, 10 clones having one individual and the other clones with four or less individuals.

Relation between ecological tolerance and population genetics

The three *D. stevensoni* populations BH, FC and IB, which have been tested for their salinity and temperature tolerance (Van Doninck *et al*, 2002), are genetically different since even the most similar clones from the different populations differ by more than 15 RAPD bands.

Discussion

Genetic variation in *D. stevensoni*

Previous surveys of population genetic variation in *D. stevensoni* reported limited allozyme variation with seven different genotypes in 36 populations and 79% of the individuals belonging to the same genotype (Rossi *et al*, 1998). However, this result was based on only two enzyme systems of which only *Gpi1* was polymorphic. Yet, using this marker, 14 European populations were multiclonal. Similarly, Havel and Hebert (1993) reported two allozyme clones in a single population from

Table 2 Mean values of N_c, N_c/n, G_0 and G_0/N_c (over seven populations) in *D. stevensoni*, minimum and maximum values in parentheses

Species	*Pop*	N_c	N_c/n	G_0	G_0/N_c
Darwinula stevensoni	7	4.7 (1–14)	0.214 (0.048–0.56)	2.91 (1–7.53)	0.776 (0.54–1)
Asexual species	1–55	1–8.67	0.04–0.46	1–5.63	0.47–1

Pop = number of analysed populations. For comparison, ranges of the means of the genotypic diversity measures of other asexual populations are given.

Northern America using one polymorphic locus, *Pgm*. Direct DNA sequencing suggested a complete lack of variation in the nuclear ribosomal ITS1 region of *D. stevensoni* over a geographical range from Finland to South Africa, based on 56 individuals from 19 populations. Some albeit low genetic variation was detected in the mitochondrial COI region (Schön *et al*, 1998). Recently, Schön and Martens (2003) reported low genetic diversity within and between *D. stevensoni* individuals using three nuclear regions ITS (see also Gandolfi *et al*, 2001), *hsp82* (exon and intron region) and a Calmodulin intron, providing no evidence of the Meselson effect.

In this study, clonal diversity was detected in seven populations of *D. stevensoni* using RAPD-PCR. All of the 33 observed clonal genotypes of *D. stevensoni* were restricted to single populations, indicating the absence of a common widespread (dominant) clonal lineage. Moreover, clonal similarity was high within compared to similarity between populations, that is, within each population clones differed only by single RAPD bands.

The genetic origin of this RAPD variation is not known. However, following Aagaard *et al* (1995) RAPD variation is most likely caused by noncoding and repetitive DNA, indicating neutral variation, whereas allozymes or coding DNA sequences represent variation in functional genes. As a consequence, because selection acts more strongly on genes encoding proteins, there is a supposedly weaker selective constraint on RAPD. This may explain why RAPD often detects more variation than techniques involving coding genes only (Rossi *et al*, 1998; Vandewoestijne and Baguette, 2002) and may be partly an explanation for the lower genetic variation reported in previous genetic studies of *D. stevensoni* (see above).

The present study was designed to investigate overall genotypic diversity in *D. stevensoni* by using RAPDs because allozymes were not a successful approach (see Rossi *et al*, 1998). However, since RAPD behave as dominant/recessive markers, bands can only be scored for presence/absence but homologous alleles cannot be assigned. Therefore, evaluating the Meselson effect is not possible from RAPD data. The latter effect can be tested by using DNA sequence data (see Mark Welch and Meselson, 2000; Schön and Martens, 2003).

Clonal diversity in asexual populations

Using published data to recalculate indices of clonal diversities from asexual populations from different species (the complete review with the recalculated data is available from KVD upon request) we found that irrespective the markers used, the indices (N_c, N_c/n, G_0, G_0/N_c) vary within roughly similar ranges. The results also indicate that most asexuals (80%) consist of multiclonal populations. The observed clonal variation in those asexual species has been mainly attributed to polyphyletic origins and asexual/sexual hybridisation (Little and Hebert, 1994; Schön *et al*, 2000 and references therein). For *D. stevensoni* the indices of clonal diversity follow the general trend of the literature (see Table 2) although it is assumed that only the accumulation of mutations is responsible for the observed variation, given that this species is supposed to be an ancient, obligatory asexual without a sexual root.

The clustering of clonal genotypes by region and the fact that clonal genotypes from the same populations tend to be genetically more similar to each other than to those from different populations, suggest that *D. stevensoni* 'clones' originate locally and do not seem to disperse much. This hypothesis fits well with the observation that *D. stevensoni* lacks dry-resistant eggs, which could be dispersed easily.

Relationship between GPG and clonal diversity

The present data suggest that the high ecological tolerance of *D. stevensoni* reported by Van Doninck *et al* (2002) is not associated with a single widespread 'clone', but involves at least four different local clonal genotypes that are ecologically highly tolerant. Hence, the prediction of the current GPG concept, that is that the wide ecological tolerance should be associated with a single widespread clonal lineage, is not corroborated here.

Recently, a series of papers on intraclonal genetic variation were published in a special issue of the Biological Journal of the Linnean Society (2003, 79). One of these by Loxdale and Lushai (2003) discusses the question 'what is a clone?'. Two definitions were given: firstly, a clone is an asexual lineage derived from a single mother; the second definition adds furthermore strict genetic, clonal fidelity. Loxdale and Lushai (2003), however, doubt the existence of strict clonal fidelity. Instead, a clone is a function of marker resolution and if sufficient regions of the genome are explored, each individual belonging to the same clone might eventually differ at some genomic regions. Keeping this in mind we propose to refine the GPG concept accordingly. A GPG is a single functional genotype producing a phenotype with a wide ecological tolerance but which may accumulate mutations in genomic regions that do not directly contribute to the phenotypic characters providing this broad ecological tolerance. In this view, the GPG concept should not be regarded as a genome-wide, fixed genotype, but rather as a specific genetic setup of a series of ecologically relevant genes.

In the absence of ecological differentiation between clones, the observed clonal diversity of *D. stevensoni* is then assumed to be the result of a balance between input of variation and stochastic extinction caused by genetic drift, the latter being determined by population size. This may be considered equivalent to the proposed GPG concept; that is, a broadly-adapted genotype that has accumulated neutral variation since the spread of the most recent advantageous mutation (Butlin *et al*, 1998).

Finally, the most challenging implication of the proposed GPG concept is that any test of the GPG based on variation at (nearly) neutral genetic markers cannot be conclusive.

Conclusion

The present work suggests that, (1) the asexual ostracod *D. stevensoni* shows genotypic variation within and between geographic populations; (2) no clonal lineage is dominant and widespread and (3) the amount and structuring of the clonal variation may still be consistent with the interpretation of *D. stevensoni* as a GPG, if the GPG is no longer considered as a genome wide phenomenon.

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