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Intraindividual and intraspecies variability of ITS1 sequences in the ancient asexual *Darwinula stevensoni* (Crustacea: Ostracoda)

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The lack of variability in ITS regions within individuals and within species has been explained as the result of concerted evolution. In fact, many examples of intraindividual variation in the ITS regions have been reported. Here we report evidence of within-individual variation of the ITS1 region in the obligate parthenogenetic species *Darwinula stevensoni*. We analysed 46 clones obtained from 12 individuals of *D. stevensoni*, from three Italian sites and one site in Luxembourg. Seven nucleotides out of 366 were variable. Most variability (80%) was found among clones within individuals, and the remainder of the variability was observed among individuals. No difference was found among populations or between habitats. The low intraspecific variability and the observation of recombinant molecules are evaluated in light of the relevant literature. The high percentage of variation within individuals and the occurrence of recombination without meiosis are discussed by considering the ancient asexual 'status' of the species.

Keywords: ancient asexual, concerted evolution, *Darwinula stevensoni*, intraindividual variability, rDNA ITS1, recombination.

Introduction

The key roles of sex in preventing the accumulation of deleterious mutations and in producing new combinations of genes which allow adaptation to changing environments (biotic and abiotic) are often indicated as a likely explanation for the prevalence of sex, despite its two-fold cost (see review by Butlin *et al*., 1998a). The existence of taxa that have apparently reproduced asexually for a long time ('ancient asexuals') has been presented as a puzzling challenge to these prevailing views (Judson & Normark, 1996; Butlin *et al*., 1998a). In a number of supposed ancient asexuals previously overlooked evidence of recombination has been found (Little & Hebert, 1996).

Darwinula stevensoni is a freshwater ostracod, considered representative of all extant species in the Darwinuloidea, because it is the only cosmopolitan species in this group. The Darwinuloidea, with bdelloid rotifers, are considered exceptions to the expected short evolutionary life span typical of asexual species: so unusual that they have been labelled 'ancient asexual scandals' (Judson & Normark, 1996). *Darwinula stevensoni* is believed to have reproduced by parthenogenesis for at least 25 My (Schön *et al*., 1998). The species is considered apomictic, even if no direct proof exists (Butlin *et al*., 1998b).

In a study of *D. stevensoni* collected from lentic habitats from South Africa to Finland, Schön *et al*. (1998) reported complete homogeneity of the ITS1 region of the ribosomal DNA, both within and among individuals, but a significantly higher variability in the COI region of the mitochondrial DNA. Allozyme data from the same populations also revealed very low variability among and within populations, with low heterozygosity values (Rossi *et al*., 1998). Havel & Hebert (1993) reported only one polymorphic allozyme out of nine screened in a single population from North America. Against this background of genetic homogeneity surprisingly different genotypic frequencies were found in Italian riverine habitats (V. Rossi, unpublished data).

It has been argued that the persistence of obligately asexual lineages could have two explanations: a highly efficient DNA damage repair mechanism or the opposite, i.e. its almost complete ineffectiveness that leads to the elimination of individuals that accumulate harmful mutations (Gabriel *et al*., 1993). Schön & Martens (1998) indicated highly efficient DNA repair as the most likely explanation for the lack of intra- and interindividual

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variability in the ITS1 region of *D. stevensoni*. This explanation has been accepted in the literature (Judson & Normark, 2000) although other possible causes have been proposed. Gene conversion with its homogenizing effect would prevent mutation accumulation (Butlin, 2000) and the accumulation of the allelic differentiation that might be detected by the so-called 'Meselson method' (Judson & Normark, 1996; Mark Welch & Meselson, 2000). Gene conversion has also been invoked as a cause of homozygosity at allozyme loci (Sywula, 1989) and could be the source of the observed low heterozygosity found in *Darwinula*, an unexpected result in an ancient asexual.

Ribosomal DNA is characterized by multiple tandemly repeated copies of highly conserved regions coding for ribosomal RNA, interrupted by variable nontranscribed 'intergenic' regions (IGS) and less variable external and internal transcribed 'spacer' regions (ETS, ITS1 and ITS2). It has been assumed, and it is still generally accepted, that the nucleotide sequence homogeneity reported among rDNA multigene families is maintained within individuals and even within species by 'concerted evolution', a phenomenon that is the result of mechanisms such as unequal crossing-over and biased gene conversion (e.g. Dover, 1982).

In spite of the fact that intraindividual and intraspecies variability is often considered to be absent, a certain degree of intraindividual variation has been found whenever it has been looked for within the ITS1 and ITS2 regions (Harris & Crandall, 2000). In fact, many exceptions exist to the rule of gene homogenization among rDNA multigene families for a vast range of organisms (Powers *et al*., 1997; Lanfranco *et al*., 1999; Harris & Crandall, 2000). This sort of heterogeneity is all the more to be expected in asexual lineages, where concerted evolution could be impeded or slowed down by the absence of recombination (Fuertes Aguilar *et al*., 1999). After a long series of apomictic generations, most sequence differences among alleles are likely to have been acquired after sexual reproduction was lost (Birky, 1996). If concerted evolution works by the occasional spread and fixation of new repeat types, at least some sequence variation of different evolutionary lineages is expected, as in different and diverging species. If concerted evolution is slow or absent, variation should be found within individuals too.

Here we report the analysis of ITS1 sequence data in individuals of *D. stevensoni* from two habitat types, lakes and rivers. If no intraindividual variation was present, a few sequences would be enough to compare the sequence data for river individuals with the "ubiquitous" sequence found in lakes by Schön *et al*. (1998) and to test, by different markers, the habitat segregation of different genetic lineages found using allozymes (V. Rossi, unpublished data). The discovery of intraindividual variation and of no sequence divergence between *Darwinula* from different habitats prompted us to focus on the discussion of the evolutionary significance of our data.

Materials and methods

Sampling

Individuals were sampled from four populations: from Angeli (Mantova lake), Monate lake and Rivalta (Mincio river) in northern Italy, and from a pond in a gravel pit in the Moselle valley (near Remerschen) in Luxembourg. Specimens were carefully washed and starved for at least 48 h prior to DNA extraction.

DNA extraction and sequencing

Genomic DNA was individually extracted from four specimens from Angeli (Ag16, Ag17, Ag18 and Ag22), two from Rivalta (Ri12 and Ri13), three from Monate (Mo06, Mo07 and Mo08) and three from Luxembourg (Lx08, Lx09 and Lx10).

Total genomic DNA was isolated with a modification (double volumes) of the 'grinding method' (CTAB lysis, chloroform: isoamyl alcohol extraction) described by Lin & Walker (1996), followed by RNAase (10 ng/μL final concentration) digestion. No-DNA extractions were also conducted to check for contamination. Concentration and integrity of the extracted DNA was determined by electrophoresis on a 0.8% agarose gel.

PCR amplifications were carried out in 25 μL volumes, using 0.1 ng genomic DNA template, 0.2 mM dATP, dCTP, dGTP and dTTP each, 0.2 μM each primer ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White *et al*., 1990) and ITS Y (5′-GTTCAAACTG-TGGCGGTTTT-3′) (Schön *et al*., 1998), 1× PCR reaction buffer (with $MgCl_2$: 1.5 mM final) and 1 unit of Taq DNA polymerase (Roche). The temperature profile was 5 min at 95°C, 36 cycles of 1 min at 95°C, 1 min at 55°C and 2 min at 72°C, followed by a final extension of 10 min at 72°C, on a PTC-100 programmable thermal controller (MJ Research). PCR products were separated by electrophoresis in a 2% agarose gel and visualized under UV light after staining with ethidium bromide.

The fragment of interest was excised from the gel and recovered following Lau & Sheu (1992). Purified PCR products were ligated into the vector pGEM-T Easy Vector (Promega) according to the instructions of the manufacturer, and subsequently transformed into competent cells (Subcloning Efficiency DH5α Competent

Cells, Gibco BRL). Plasmid DNA was isolated according to a miniprep protocol (Sambrook *et al*., 1989).

Plasmids from single colonies were sequenced in both directions using an ALFexpress sequencing system (Pharmacia Biotech), with primers M13 universal and M13 reverse (Amersham Pharmacia Biotech). Standard protocols of the manufacturer for Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) were followed.

The sequence for each clone was determined by comparing the two single strand chromatograms, using CHROMAS software (http://www.technelysium.com.au/chromas.html).

Sequence analysis

Sequences from different clones and individuals were aligned to the only sequence found by Schön *et al*. (1998) (GenBank accession number AF031305) using CLUSTAL X (Thompson *et al*., 1997) and checked manually. As no insertions/deletions were found within the species, gap weight did not influence the alignment. The presence or absence of both universal and species-specific primers within the sequences was checked, and the boundaries of the ITS1 sequence were determined by comparison with those of other organisms in the GenBank database. To make our data comparable with the literature, genetic distances among sequences were estimated as mean gamma distance using a Kimura two-parameter model ($a = 1$) with MEGA 1.02 (Kumar *et al*., 1993). We also used the same method with gamma a values of 0.5, 500, and 1000, the Proportion of Difference method and the Jukes and Cantor method. Mean nucleotide diversity (π) was calculated from pairwise genetic distances following Nei (1987).

Unrooted trees were obtained by maximum likelihood and by UPGMA analysis of distances estimated by Kimura's two-parameter method (Kimura, 1980), using 1000 bootstrap replicates, with PHYLIP package software (Felsenstein, 1989). A network showing all the possible mutation steps among sequences was drawn by hand and checked by TCS software (Clement *et al*., 2000).

Analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) was used to hierarchically partition genetic diversity: within individuals, among individuals within populations (or habitats), and between populations (or habitats). Analysis of the distribution of genetic variance was performed by ARLEQUIN 2.0 software (Schneider *et al*., 1997) based on a distance matrix computed by Kimura's two-parameter method, with gamma $a = 1$, and significance tested using 10000 permutations (all remaining parameters given default values).

Results

DNA sequences (366 bp long) for 46 clones from 12 individuals of *D. stevensoni* from four different populations were obtained (GenBank accession numbers AF305009–AF305054). The 320 bp sequence reported as part of the ITS1 region by Schön *et al*. (1998) was identical to the sequence we found in clones Ag22c.2, c.3, c.4 and Ri12c.3 (from nucleotide 49 to nucleotide 366, plus two adenines that we assumed to be the last two nucleotides at the 3′ position of the primer ITS Y). A comparison with GenBank database sequences indicated that the whole sequenced region corresponds to a partial sequence of the 18S subunit, a complete sequence of the ITS 1 region and a partial sequence of the 5.8S subunit. The first 29 bases after primer ITS 5 and the last 75 bases before primer ITS Y are highly conserved even among evolutionarily distant species. The effective size of the ITS 1 region in *D. stevensoni* should consequently be 262 bp, but all 366 nucleotides were considered in the analyses. No variation in length among different sequences was observed.

The universal primer ITS 1 (White *et al*., 1990) annealing region was completely conserved in *D. stevensoni*, whereas the universal primer ITS 2 annealing region (equal to primer ITS 3 complemented and reversed) (White *et al*., 1990) presented some nonconserved bases, particularly at the 3′ position. This divergence may explain the difficulty in amplifying the ITS 1 region with universal primers. The species-specific primer ITS 1D, designed by Schön *et al*. (1998), was found to be lacking a cytosine (base 34).

Thirteen nucleotides out of 366 were polymorphic within or among individuals (Fig. 1). Point mutations at seven sites were present in at least a single repeat in different individuals. The remaining six mutations were observed in single clones. As it is possible that some of these six mutations are the result of mistakes introduced by Taq polymerase during amplification, the five sequence variants showing these mutations were excluded from the analyses.

The remaining 41 clones yielded 11 distinct sequences with at least two variants per individual (except for Ag16). Eight sequence types were found in two or more individuals (Fig. 1). Overall, mean and maximum divergences among different clones were 0.5 and 1.4%, respectively (Table 1). A very similar range of divergences was observed by comparing sequences within individuals (Table 1). Genetic divergences estimated with different methods gave very similar values (data not shown).

AMOVA analysis confirmed that most of the variation (80%) occurred within individuals (Table 2). The among-populations variance was negative but not significantly

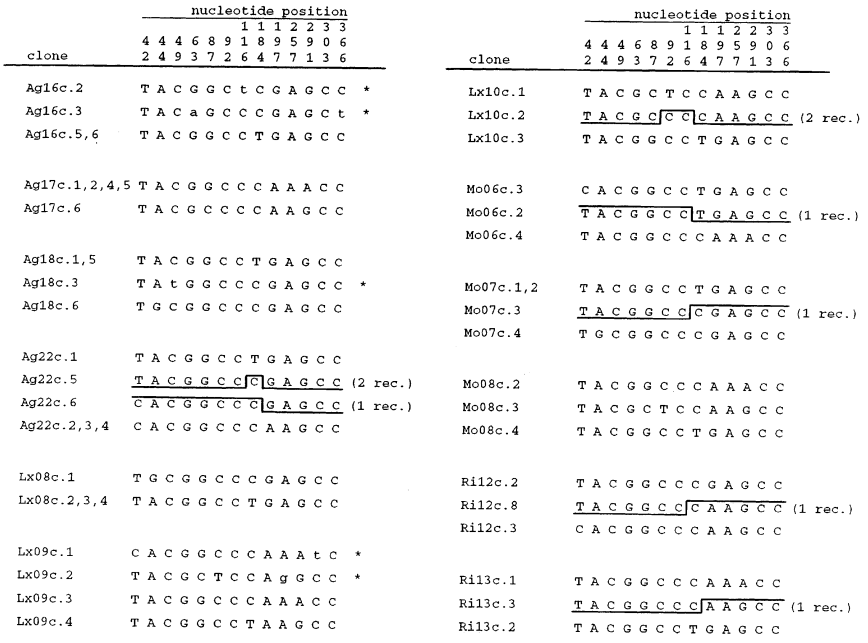

Fig. 1 Polymorphic sites per clone per individual of *Darwinula stevensoni*. Asterisks denote sequence variants ignored in the analyses because they presented mutations seen only in a single clone. Possible recombinants: 2 rec., double recombination event; 1 rec., single recombination event. AgXc.Y: clone Y of individual X from Angeli; RiXc.Y: clone Y of individual X from Rivalta; MoXc.Y: clone Y of individual X from Monate; LxXc.Y: clone Y of individual X from Luxembourg.

Table 1 Nucleotide diversity in *Darwinula stevensoni* expressed as mean (= π in Nei, 1987) and maximum (in parenthesis) values of gamma distances estimated by a Kimura two-parameter model

Hierarchic levels	Repeated polymorphisms	Repeated sequences	Repeated & non- recombinant
Overall	0.005 (0.014)	0.005 (0.011)	0.006 (0.011)
Among populations	0.005 (0.014)	0.005 (0.011)	0.006 (0.011)
Among individuals	0.006 (0.014)	0.006 (0.011)	0.006 (0.011)
Within individuals	0.004 (0.011)	0.004 (0.011)	0.004 (0.011)

Computations were performed by considering different hierarchical levels and different sequence sets (repeated polymorphisms: sequences showing at each polymorphic site only nucleotides found in at least two independent clones; repeated sequences: sequences found in at least two independent clones; nonrecombinant: sequences not derived from a possible recombination between other sequences observed. Different estimates (e.g. proportion of difference or Jukes & Cantor methods) gave very similar values (not shown).

Table 2 Hierarchical analysis of genetic variance using AMOVA among populations, among individuals within populations, and within individuals of *Darwinula stevensoni*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	p	±
Among populations	3	2.22	−0.12	−12.05	0.989	0.001
Among individuals within populations	8	15.00	0.32	32.22	0.001	0.000
Within individuals	29	23.14	0.80	79.83	0.004	0.001
Total	40	40.36	1.00			

Significance tested using 10000 permutations. In the 'among populations' and 'among individuals within populations' sources of variation: p = P(random value ≥ observed value); in the 'within individuals' source of variation: p = P(random value ≤ observed value).

different from zero. Negative variance components usually indicate an absence of genetic structure, although the source of variation can have a biological meaning (Excoffier, http://lgb.unige.ch/arlequin). The analysis, repeated without considering the 'among-populations' level, gave the same pattern: most of the variation (78%) was found within individuals. The same picture (82%) emerges by considering habitats instead of populations as the highest hierarchical level.

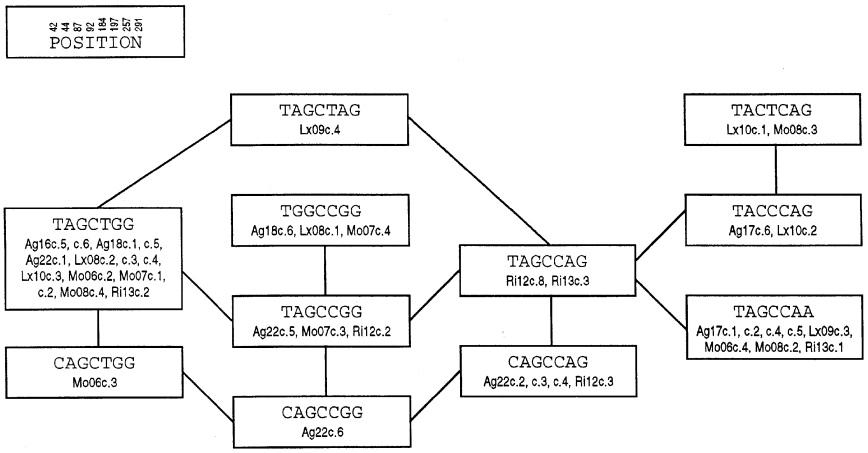

Fig. 2 For each observed sequence variant in *Darwinula stevensoni* the state of variable sites is shown. Clones of different individuals and populations that presented a variant are indicated below the sequence. Each line connecting two sequences represents a single mutation.

Whichever method is used, a single tree with stable nodes cannot be constructed. The possible mutation steps that led to the observed variation among sequence variants can however be represented as a network (Fig. 2). Sequences Ag22c.6, Mo06c.2, Mo07c.3, Ri12c.8 and Ri13c.3 are consistent with the hypothesis that they are 'recombinants' of other clones of the same individuals, and Ag22c.5 and Lx10c.2 could be double recombinants (Fig. 1).

Discussion

We found intraindividual variation in ITS1 sequences of *D. stevensoni*. This is in contrast with the findings of Schön *et al.* (1998). Given the small number of clones examined per individual, it could be argued that we detected only a fraction of the ITS1 variants present in the genome (van Herwerden *et al.*, 1999). The observation of identical sequences in different organisms could be an effect of preferential amplification of a few repeat types favoured by peculiar PCR conditions (see e.g. Fenton *et al.*, 1998). A dominance effect in PCR could also be a possible explanation for the homogeneity found by Schön *et al.* (1998). The presence of within-individual heterogeneity eliminates the need to invoke highly efficient repair mechanisms proposed to explain the lack of sequence variability (Schön & Martens, 1998; Judson & Normark, 2000).

Our results support the recent observation by Harris & Crandall (2000) that intragenomic variation in ITS1 and ITS2 is common although widely ignored. Low variability, mostly found within individuals, has often been reported for ribosomal DNA internal spacers: in nematodes (Blok *et al.*, 1998), corn rootworm beetle (Szalanski *et al.*, 1999), mites (Navajas *et al.*, 1999) and *Anopheles nuneztovari* (Fritz *et al.*, 1994). In *Darwinula*, Schön *et al.* (1998) reported distance values for mitochondrial sequences (COI) within (0.4–0.6%) and between (2.2%) two groups of European populations similar to what we measured using ITS1. A possible explanation for ITS1's low diversity might be the spacers' role in rRNA secondary structure, of key importance in post-transcriptional processing (Lalev & Nazar, 1999).

Most variability is found at the intraindividual level (80%), whereas no difference was found among populations or between habitats. Consequently, the segregation of isozyme clones between lacustrine and riverine habitats (V. Rossi, unpublished data) could not be confirmed, although this conclusion is tentative because of the small number of individuals we analysed.

Concerted evolution among rDNA copies has not been extensively investigated in apomictic clonal lineages (Birky, 1996; Hugall *et al.*, 1999): although theoretically possible, it seems to be slowed down or even halted (Fuertes Aguilar *et al.*, 1999). The absence of meiosis limits the formation of heteroduplex molecules that are required for efficient repeat homogenization (Campbell *et al.*, 1997). According to Lanfranco *et al.* (1999) heterogeneity of ribosomal sequences in mycorrhizal fungi, thought to be asexual organisms, could be explained by the absence of mechanisms associated with recombination and leading to concerted evolution (but see 'note in proof' in Normark, 1999). Van Herwerden *et al.* (1999) found intraindividual variability both in diploid and triploid parthenogenetic forms of *Paragonimus westermani* (Trematoda). Hugall *et al.* (1999) observed high intraindividual variability in four apomictic nematodes but no variation within individuals of two amphimictic species. Contrasting results were found by Crease & Lynch (1991), who observed a lower mean number of rDNA repeat types in obligately apomictic clone isolates of *Daphnia pulex* than in their cyclic relatives.

Eight of the different sequences we obtained from clones could be the result of recombination, although

we can not exclude that they are PCR artefacts. Recombinant amplification products can be produced *in vitro*, as a consequence of 'jumping' PCR, particularly when amplifications are initiated from single or very few template molecules and the target DNA is represented by repetitive sequences and genes belonging to multigene families (Campbell *et al*., 1997; Fuertes Aguilar *et al*., 1999; Hugall *et al*., 1999). However a double *in vitro* recombination in the case of sequences Ag22c.5 and Lx10c.2 seems unlikely. Moreover, all sequences from the potential *in vitro* recombinants (with the exception of clone Ag22c.6) were found in other clones from different individuals. Sequences Ri12c.8 and Ri13c.3 are identical, and it is unlikely that they originated from two independent artefactual amplifications. The sequence variants that we consider to result from recombination may be produced by crossing-over (single recombinants) or 'partial' gene conversion (a more likely explanation for double recombinants).

Gene conversion can occur in the absence of meiosis and can prevent allelic divergence among multigene families as well as between alleles at a single locus (Normark, 1999). Crease & Lynch (1991) showed that recombination occurs within and between *Daphnia*'s rDNA arrays in the absence of sexual reproduction, leading to a loss of variation in obligately parthenogenetic clones. Such a DNA homogenization process would have important evolutionary implications for obligate parthenogens. Butlin (2000) proposed gene conversion as the most likely explanation for the ITS1 homogeneity in *D. stevensoni* (Schön *et al*., 1998) and suggested it would permit the existence of the ancient asexuals by eliminating accumulation of mutations.

The low variability we found could support this hypothesis. However, the finding of new repeats derived from recombination indicates that new variants may be produced not only by mutation and fixation. Gene conversion, if slow enough, and crossing over could lead to a result completely different from the expected sequence homogeneity. New mutations could occasionally spread to variants other than the ones in which they originated, and join with other mutations present in regions not involved in the recombination process. This would accelerate the evolutionary opportunities of parthenogenetic organisms by combining favourable mutations, just as in sexual recombination.

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