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Persistence of asexuality through mixed reproduction in *Eucypris virens* (Crustacea, Ostracoda)

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The ostracod species *Eucypris virens* exhibits geographical parthenogenesis, with rare sexual populations in southern Europe and widespread asexual populations elsewhere. DNA sequence data from the nuclear ITS1 and mitochondrial COI regions have been used to estimate genetic variabilities and reconstruct phylogenies. The observed divergence was exceptionally high, with intraspecific maxima of 10.3% (ITS1) and 20.9% (COI) among European lineages, levels reported for interspecific comparisons of other taxa. Phylogenetic reconstructions reveal multiple origins of asexual clones from sexual populations. However, we argue that such data can only provide a lower limit on the number of origins of asexual reproduction, and an upper limit on the age of asexual lineages. Congruence between gene trees for different loci can provide support for the inference of long-term apomictic reproduction. Nuclear and mitochondrial data differ in their placement of some asexual clones, possibly indicating that genetic exchange has taken place between sexual and asexual lineages. Such intraspecific hybridization is one route to combine the benefits of both reproductive modes, and it might explain how asexuality managed to persist in *E. virens* even in long, evolutionary terms.

Keywords: asexuality, COI, ITS1, Ostracoda.

Introduction

The influence of reproductive mode on the persistence and genetic divergence of lineages has been discussed for decades (Maynard Smith, 1978) and is still the object of extended debate (Hurst & Peck, 1996; Martens, 1998). Asexual reproduction might have evolutionary advantages in the short term: coadapted gene combinations are not separated through meiosis, the exclusive production of female offspring saves up to half of the total energetic costs of reproduction, and new habitats might be colonized successfully by a single female (Maynard Smith, 1978). However, there are several disadvantages in the longer term, finally leading to extinction: asexuals are supposed to accumulate deleterious mutations, lose uncorrupted genotypes by chance and be unable to evolve in response to rapid environmental changes.

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Asexuality exists in many different taxonomic groups and is relatively common in freshwater ostracods (Martens, 1998). With the exception of a few 'ancient asexuals' (Butlin *et al*., 1998; Schön *et al*., 1998), the distribution of asexuality probably reflects a balance between repeated origin of asexual lineages and their extinction when the long-term costs of asexual reproduction outweigh the short-term benefits (Butlin *et al*., 1998). The debate becomes more complex when asexual and sexual reproduction are not exclusive alternatives. Mixed reproduction could turn out to be the most beneficial evolutionary path and theoretical considerations (Hurst & Peck, 1996) suggest that 'a little sex now and then' will be sufficient to avoid the accumulation of mutations and to maintain genetic variability.

Several strategies are known, where the two reproductive modes are alternated or combined, e.g. cyclic parthenogenesis (as in *Daphnia*), gynogenesis or hybridogenesis as in asexual vertebrates (Avise *et al*., 1992), and the occurrence of rare males (Martens,

1998). We mainly focus on the impacts of hybridization, as this seems to be important for maintaining asexuality in nonmarine ostracods (Chaplin *et al*., 1994; Butlin *et al*., 1998). Asexual females have been found to hybridize with males from the same or closely related species (Turgeon & Hebert, 1995) leading to the formation of new (mostly polyploid) clones, and polyploidy is widespread in this group (Tétart, 1978; Chaplin *et al*., 1994).

Potential hybrids can be identified, if sequence data from nuclear and mitochondrial DNA are used for phylogenetic constructions. This approach revealed that all asexual vertebrates thus far analysed are the result of interspecific hybridization (Avise *et al*., 1992).

DNA sequence data can, furthermore, be used to estimate the age of asexual lineages by applying the principle of the molecular clock. Although age estimates are essential to test the theoretical prediction that asexuals are doomed to extinction in the long term, such data are still rare for asexual invertebrates. Perez *et al*. (1994) concluded that two parthenogenetic lineages of *Artemia* might have existed for 30–40 Myr, whereas other studies have analysed the mitochondrial DNA of freshwater ostracods, not by sequencing, however, but by restriction digestions (e.g. Chaplin & Hebert, 1997). The latter estimated that asexual clones of *Heterocypris incongruens* (as *Cyprinotus incongruens*; nomenclature throughout follows Meisch *et al*., 1989) could have existed for 5 Myr.

Allozyme data show that asexual lineages of freshwater ostracods can be exceptionally clonally diverse (Chaplin *et al*., 1994; Butlin *et al*., 1998). The highest clonal diversity of freshwater ostracods so far has been found in *Eucypris virens* (Rossi *et al*., 1998), a Holarctic species, which prefers temporary or semipermanent ponds. Although asexually reproducing *E. virens* occurs throughout Europe, sexual populations have been described from only a few localities around the Mediterranean area (Martens, 1998), a phenomenon known as geographical parthenogenesis (Vandel, 1928). Asexual populations appear, from allozyme analysis of laboratory cultures, to be exclusively apomictic (V. Rossi, pers. comm.).

We present molecular data on the genetic variability of *E. virens* from nuclear and mitochondrial DNA. Sequence data are used to reconstruct phylogenetic relationships among populations of different geographical origins and/or reproductive modes. In order to explain the exceptionally high genetic divergence, we aim to test whether asexuality has a multiple origin in this species, whether asexual lineages have existed for a long time, and whether there is evidence for the combination of both reproductive modes through hybridization.

Materials and methods

Species and populations

Specimens of *E. virens* were collected from 14 different European sites (Table 1). Twelve sites had all-female populations though males were present at the Extremadura (Spain) and Sicily (Italy) sites. Allozyme data (Rossi *et al*., 1998) indicate that the Sicilian population is exclusively sexually reproducing, whereas the Extremadura site includes populations with both reproductive modes. Therefore, at Extremadura, males are taken to represent the sexual (sub)population whereas individual females may come from either subpopulation. Samples of *Prionocypris aragonica* (an Iberian endemic of the Eucypridinae) and British *P. zenkeri* (Eucypridinae) were included as outgroups.

DNA extraction, PCR and automatic sequencing

DNA was extracted from each individual separately as described elsewhere (Schön *et al*., 1998). 'No-DNA' extractions were conducted and carried through all stages of processing to check for contamination.

Table 1 Number and origin of samples analysed for *Eucypris virens*

Site	Abbreviation	*N*†	Reproductive mode‡
Extremadura, Spain	Extr	8/6§	asex/sex
Pico, Spain	Pico	4/3	asex
Berzosa, Spain	Berz	3/0	asex
Morcuera, Spain	Morc	1/0	asex
Code d'Asino, Italy	Asin	1/0	asex
Biglinia, Italy	Bigl	1/0	asex
Sanguinaro, Italy	Sang	3/3	asex
Sicily, Italy	Sici	7/1¶	sex
Colorno, Italy	Colo	0/1	asex
Ventina, Italy	Vent	3/3	asex
Leeds, UK	Leed	1/1	asex
Bramhope, UK	Bram	3/0	asex
Ketton, UK	Kett	1/0	asex
Haantjen, Belgium	Haan	2/0	asex

† *N*, number of individuals screened for ITS1/COI.
‡ asex, asexual; sex, sexual reproduction.
§ Extremadura males: four for ITS and three for COI; females: four for ITS and three for COI.
¶ Sicily: one male for ITS, six females for ITS; one male only for COI.
Coordinates of the sample sites are available from the first author on request.
Three specimens of each outgroup were analysed for ITS; only *Prionocypris aragonica* for COI.

Internal transcribed spacer (ITS1)

The universal primers ITS1 and ITS4, which were designed for the conserved parts of the 18S and 28S ribosomal genes (White *et al*., 1990), were used initially to amplify the whole ITS region. Sequence data from the approximately 1000-bp PCR product were used to design a species-specific internal primer, ITSX, which improved the quality and reliability of both PCR and sequencing. With primers ITS1 and ITSX, a 350-bp-long part of ITS1 was amplified. We checked the identity of the sequences by a BLAST search: the sequences from *E. virens* aligned with ITS1 from Chironomidae and the distantly related ostracod *Darwinula stevensoni* (Schön *et al*., 1998) and with ribosomal DNA from other invertebrates. (No ITS sequences from other crustaceans have been published yet.)

PCR amplifications were carried out in 25 μL volumes, using 4–6 μL DNA-extraction mix, 200 μM dNTPs, 1 mM Tris/HCl, pH 8.3, 5 mM KCl, 1.5–3 mM $MgCl_2$, 10 pmol of each primer ITSX (5′-TAAACACCCTTATCCTG-3′) or ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS1 (5′-TCCGTAGGTGAACCTGCGGAAGGAT-3′) and 0.5 U Thermoprime (Applied Biosystems). The typical temperature profile consisted of the following steps in a TrioThermoblock (Biometra): 5 min 95°C; 35 cycles of: 1 min 95°C, 1 min 50°C, 1 min 30 s 72°C; 10 min 72°C.

Cytochrome oxidase subunit I (COI)

Part of the mitochondrial COI gene was amplified by PCR. The general primers HCO2198 and LCO1490 (Folmer *et al*., 1994) were used for PCR and sequencing, until the species-specific primers HCOEI (5′-TTAATAGCATAGTAATGGCC-3′), HCOEII (5′-AGATATTCCTTGAAAGAGGAGG-3′), LCOEI (5′-GTCATTCGAGCCGAATTAGG-3′) and LCOEII (5′-ACCCTCCCCTTTCAAGAAATAT-3′) had been designed. Identity of the ostracod sequences was confirmed by alignments with COI sequences from the crustacean *Penaeus vannamei* (Genbank accession no. X82503) and the ostracod *D. stevensoni* (Schön *et al*., 1998). Primers HCOEI and LCOEI were used for PCR and sequencing, the internal primers HCOEII and LCOEII for sequencing only. Amplifications were carried out in 25 μL volumes, using 4–6 μL DNA extraction mix, 200 μM dNTPs, 1 mM Tris/HCl, pH 8.3, 5 mM KCl, 1.5–3 mM $MgCl_2$, 10 pmol of primer HCOEI and LCOEI and 0.5 U Thermoprime (Advanced Biotechnologies). The typical temperature profile consisted of the following steps in a TrioThermoblock (Biometra): 5 min 95°C; 35 cycles of: 1 min 95°C, 1 min 40°C, 2 min 72°C; 10 min 72°C.

Sequencing

PCR products were electrophoresed in 0.8% agarose gels, ethidium bromide stained, visualized under UV illumination and photographed. PCR fragments were cleaned with the Wizard DNA PureFection kit (Promega), and approximately 5 ng of the PCR product and 3.3 pmol of primer were used for cycle sequencing.

Sequencing reactions were carried out with the PRISM Dye Terminator Cycle Sequencing Reaction kit (Applied Biosystems) according to the manufacturer's protocol (25 cycles with 30 s at 96°C, 15 s at 50°C and 4 min at 60°C), cleaned, electrophoresed, and analysed with an ABI 373 (Applied Biosystems Inc.) Automated Sequencer.

Data analyses and phylogenetic reconstructions

To verify DNA sequences, the PCR product of each individual was sequenced in both directions and the automatically generated results for the two strands were aligned and checked. Intra- and interspecific alignments were conducted with PILEUP in the WISCONSIN GCG PACKAGE and are available on request from the first author. Further sequence analyses were conducted with MEGA 1.01 (Kumar *et al*., 1993). The construction of trees (with random input order and the options 'heuristic search' and 'step-wise addition') and bootstrapping with 1000 replicates were conducted with PAUP (Swofford, 1998). Trees were manipulated with MACCLADE (Maddison & Maddison, 1992) and likelihood analyses were conducted with PAML (Yang, 1996). All sequences are available on Genbank, accession nos AJ241478–AJ241539.

Results

Genetic variability

A 350-bp part of ITS1 was sequenced for 38 individuals of *E. virens* from 14 different localities throughout Europe. Distances between *E. virens* and its outgroups, and among *E. virens* from the same or different sites, were estimated with the Kimura two-parameter method, compensating for multiple hits and biases in transition/transversion ratios. The observed mean transition:transversion ratios were 0.87 between species, 2.52 between populations within species and 2.76 within populations, indicating saturation of transitions between *E. virens* and the outgroups where divergence was much higher than anticipated (Table 2). Intraspecific divergence was also remarkably high: average pairwise distances among European populations had a mean of 4.4% and a maximum of 10.3%. Divergence between individuals within populations was generally low ($< 2\%$) except for

Table 2 Mean Kimura two-parameter distances (minimum–maximum) for *Eucypris virens*, *Prionocypris aragonica* and *P. zenkeri*

		COI			
Comparison	ITS1	All	1	2	3
E.virens/ *P. aragonica*	0.352 (0.326–0.393)	0.241 (0.218–0.293)	0.106 (0.0678–0.136)	0.0332 (0.029–0.0668)	0.864 (0.705–1.22)
E.virens/ *P. zenkeri*	0.399 (0.377–0.460)				
E. virens between sites	0.0436 (0–0.103)	0.150 (0.0169–0.209)	0.0495 (0.0071–0.0999)	0.0069 (0–0.0746)	0.551 (0.0147–0.891)
E. virens within sites:					
Extremadura	0.0250 (0–0.0471)	0.111 (0.0024–0.216)	0.0568 (0–0.0994)	0.009 (0–0.0439)	0.37 (0.0073–0.738)
Berzosa	0.0185 (0.0046–0.0278)				
Pico	0.0123 (0–0.0184)	0	0	0	0
Bramhope	0.0154 (0.0092–0.0185)				
Sicily	0.0013 (0–0.0046)				
Sanguinaro	0.0031 (0–0.0046)	0.0096 (0.0048–0.0144)	0.0095 (0–0.0143)	0	0.0197 (0–0.0296)
Ventina	0	0.0048 (0.0024–0.0072)	0.0047 (0–0.0071)	0	0.0097 (0.0073–0.0146)
Haantjen	0.0233				

the population with mixed reproductive mode (Extremadura) where two individuals differed markedly from the other six (see below), and for one unusual individual from Bramhope. Average genetic distances between asexual populations were significantly correlated with geographical distances between sites (standardized Mantel coefficient $Z = 0.56$, $P < 0.01$).

Three short indels distinguished the ITS1 sequence of two Extremadura females (Extr5 and Extr6) from that of all other *E. virens* individuals. These two individuals showed high genetic distances from Extremadura males and the other two Extremadura females but were similar to one another. They most likely represent asexual lineages living sympatrically with sexual populations. The three indels are all present in at least one of the outgroup species, suggesting that they might be ancestral. Other indels separate *Eucypris* from *Prionocypris* but no other indels were detected within *Eucypris*.

Mitochondrial COI sequences of 423 bp were compared for 18 individuals from seven sites (Table 2), including 13 individuals, which had also been analysed for ITS1. As in ITS1, Kimura two-parameter distances were high between *E. virens* and *P. aragonica*, and among European *E. virens* populations, whereas variability within sites was low with the exception of the mixed, Extremadura site. Substitutions at the third codon positions (Table 2) were always the most numerous, followed by first position changes as expected, and transition: transversion ratios were high for first and third codon positions (5.17 and 5.11 within species, among population comparison, respectively) but low in second codon positions (0.70).

From those individuals analysed for both regions, a scatter plot of the estimated interindividual Kimura distances was examined to check for the expected correlation in variability of the nuclear and mitochondrial sequences (figure not shown). A Mantel test revealed a marginally significant association ($Z = 0.22$, $P = 0.04$). However, there are exceptions where the COI distances are low but ITS distances are high. These involve comparisons between the two putatively asexual individuals from Extremadura (Extr5 or Extr6) and the three specimens from Pico and are suggestive of mitochondrial gene exchange between sexual and asexual lineages, as discussed below.

Molecular phylogeny

A neighbour-joining tree constructed with Kimura two-parameter distances and based on a 348-bp alignment of

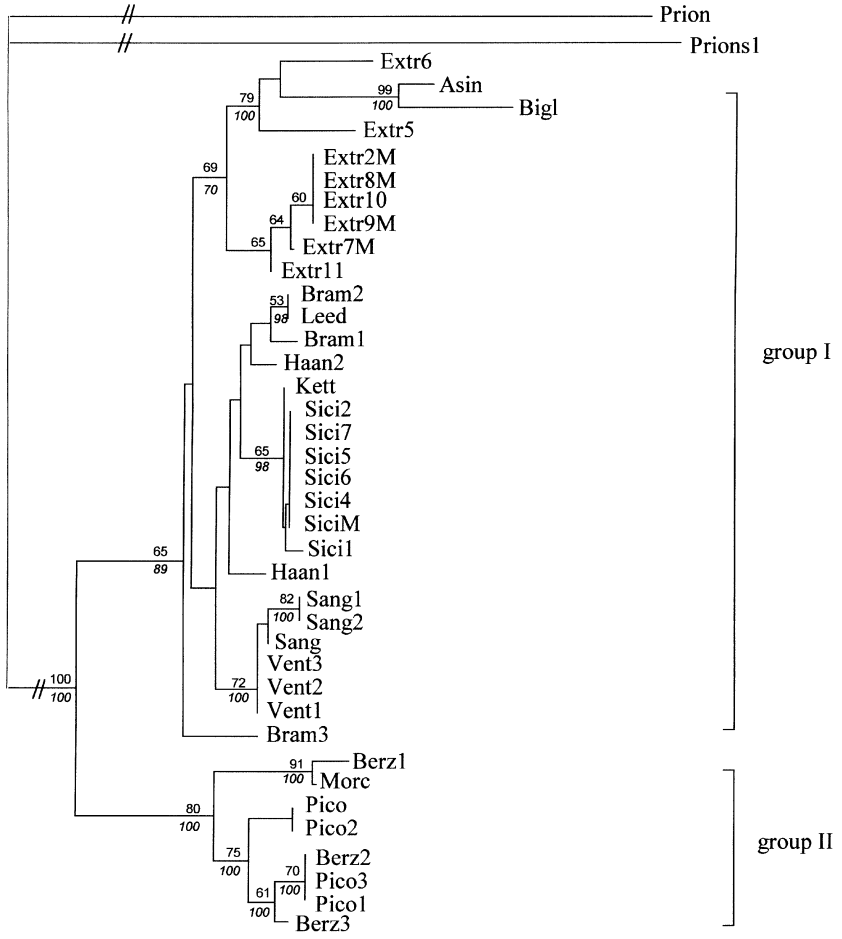

Fig. 1 Neighbour-joining tree constructed from ITS1 data for *Eucypris virens* using Kimura two-parameter distances and including all samples. Branch lengths are proportional to genetic distances, except for the outgroup branches which were shortened manually after tree construction. Bootstrap percentages, based on 1000 replicates, are given above branches for values > 50%. Values below branches are the percentage of occurrence of the branch among 132 equally parsimonious topologies of length 339. The topology shown has treelength = 344; CI (consistency index) = 0.0866; RI (retention index) = 0.834. Prion, *Prionocypris aragonica*; Prions1, *Prionocypris zenkeri*; M, male.

ITS1 sequence data reveals two major branches (Fig. 1): one (I) consists of a mixture of European populations with different reproductive modes and geographical origins, and the second (II) includes only Spanish, asexual populations. The first group lacks any deep separation according to reproductive mode: asexual and sexual populations cluster together in the same subbranch (as in the case of Sicily and the northern populations Haantjen, Ketton, Leeds and Bramhope), whereas the two sexual populations (Sicily and Extremadura) are quite distantly related. Two putatively asexual females from the Spanish Extremadura population (Extr5 and Extr6) do not group with males and other females from the same site, but instead with asexual populations from Italy. Otherwise, individual ostracods cluster according to their collection site, except for one Bramhope specimen.

Maximum parsimony analysis of the ITS1 data produced 132 equally parsimonious trees of length 339, consistency index 0.879 and retention index 0.852. These trees differ primarily in the resolution of within-site relationships but also in some deeper nodes within group I which also have low bootstrap support in the neighbour-joining tree. A semistrict consensus tree is fully compatible with the neighbour-joining tree for relationships among sites (Fig. 1), except that it places (Sang + Vent) as sister clade to (Extr + Asin + Bigl) rather than as sister to (Sici + Haan + Kett + Leed + Bram). The topology of the tree shown in Fig. 1 requires five additional substitutions.

These analyses do not take account of the information provided by indels. As noted above, three indels present in Extr5 and Extr6 might be ancestral and suggest that these individuals should branch basally. Forcing this position in parsimony analysis requires an increase in tree length of seven substitutions relative to the most parsimonious topologies, but does not alter the remaining relationships among sites.

Trees were also constructed for sequence data from the mitochondrial Cytochrome oxidase I (Fig. 2). The topologies obtained from neighbour-joining and maximum-parsimony approaches were in full agreement for branches with greater than 50% bootstrap support. However, the ITS1 and COI topologies differ in two major respects: in the COI tree the two putatively asexual females from Extremadura (Extr5 and Extr6)

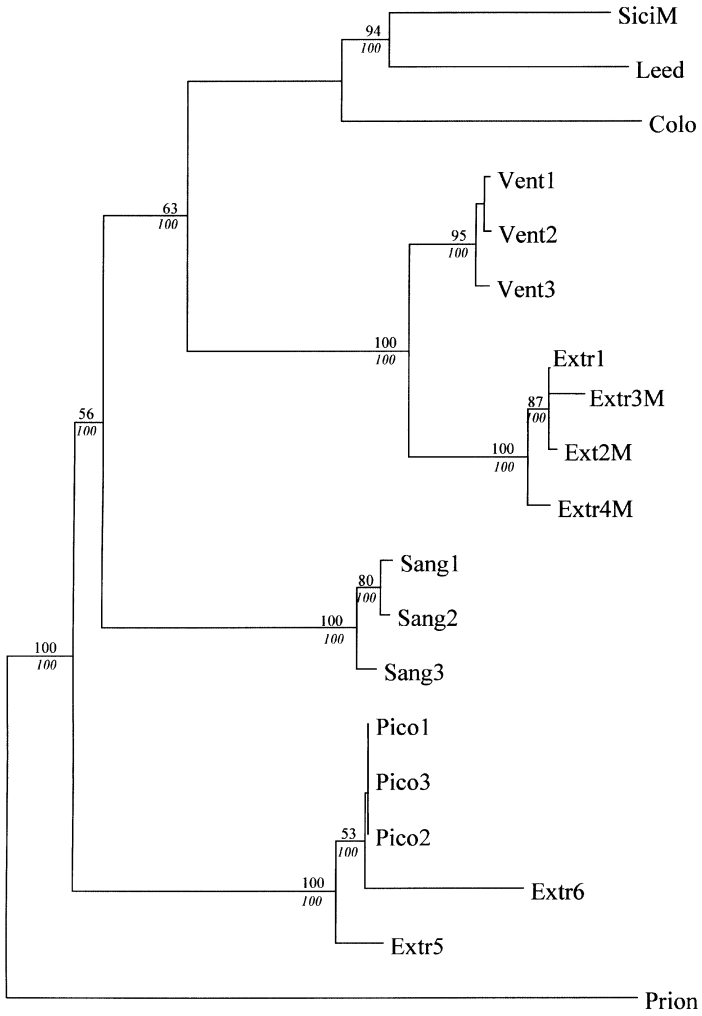

Fig. 2 Neighbour-joining tree constructed from COI data for *Eucypris virens* using Kimura two-parameter distances and including all samples. Branch lengths are proportional to genetic distances. Bootstrap percentages, based on 1000 replicates, are given above branches for values > 50%. Values below branches are the percentage of occurrence of the branch among eight equally parsimonious topologies of length 303. The topology shown has treelength = 301; CI (consistency index) = 0.6877; RI (retention index) = 0.8210. Prion, *Prionocypris aragonica*; M, male.

cluster with Pico, a Spanish asexual population that belonged to Group II in the ITS tree (as expected from the comparison of distance matrices discussed above); and Sanguinaro is basal in Group I of the COI tree rather than being sister to Ventina.

In order to compare topologies statistically, trees were also constructed from ITS1 and COI data using a congruent set of individuals. For COI, the topology of the tree (Fig. 2) was not altered by the removal of three additional specimens. The ITS1 tree for the congruent data set (not shown) differs from the COI tree in the same ways as the tree from the full data set. However, although the ITS1-based tree fits the COI data very poorly (requires 67 more substitutions and log-likelihood reduced by 112 units relative to the COI tree), the ITS1 data are adequately explained by the COI-based tree (requires six additional substitutions and log-likelihood reduced by 1.7 units relative to the ITS1 tree). This asymmetry results from the larger number of informative sites in the COI data set. Both data sets were also joined and used for phylogenetic reconstructions. Eight equally parsimonious trees were obtained, with a length of 337, a consistency index CI of 0.7454 and a homoplasy index of 0.2546. The trees are not shown, because their topology resembles the COI tree (Fig. 2).

Age estimation

If absolute ages are estimated with the molecular clock, the most serious problem is the calibration of evolutionary rates from related taxa. The only published estimate of an evolutionary rate for ITS is 1.2% per Myr for *Drosophila* (Schlötterer *et al.*, 1994). Using this value, lineages of *E. virens* from the same site would have diverged up to 1.15 Myr ago (i.e. 2.3 Myr divergence; using Berzosa data) and the split between lineages at different sites would have happened 1.8 Myr ago (on average) with a maximum of 4.3 Myr ago.

The mitochondrial COI region in crustaceans has been estimated to evolve with a rate of 1.1–1.3% per Myr per lineage (Knowlton *et al.*, 1993). For *E. virens*, we would then obtain 5.8–6.8 Myr (mean) to 8–10 Myr

(maximum) since the separation of lineages at different sites. Schön *et al*. (1998) used fossil data to estimate a substitution rate for COI in the ostracod *D. stevensoni* between 0.13 and 0.55% per Myr. Molecular evolution in the Darwinulidae is most likely slower than in other ostracods (Schön *et al*., 1998), and using these calibrations could therefore lead to overestimations.

Discussion

Genetic variability

The analysed part of the nuclear ITS1 region is extremely variable among our samples of *E. virens*. Intraspecific levels of divergence exceed those of other invertebrates, which are predominantly sexually reproducing. Less than 0.05% divergence was reported for species of *Drosophila* (Schlötterer *et al*., 1994), and 0.4% for mites (Navajas *et al*., 1994). Higher values have been described at the interspecific level, reaching for example 16% in the *Culex pipiens* complex (Miller *et al*., 1996). Only a study on the tiger beetle *Cicindella dorsalis* has shown high intraspecific ITS1 variability, but this was caused by variation among multiple copies of ITS1 within individuals (Vogler & De Salle, 1994). This explanation is rather unlikely for *E. virens*, as we obtained clear and reliable sequence data directly from amplification products (as was the case in another ostracod species; Schön *et al*., 1998).

Intraspecific variation in COI sequences is low in other arthropods such as mites (Navajas *et al*., 1994). However, in crustaceans, values of 17 and 20% were observed among *Artemia* lineages with different reproductive modes (Perez *et al*., 1994), and 18% was reached within the copepod species *Tigriopus californicus* (Burton & Lee, 1994). Another nonmarine ostracod with mixed reproductive modes (*Heterocypris incongruens*) also shows high mtDNA variability (Chaplin & Hebert, 1997).

An exceptionally high, genetic divergence of *E. virens* is also supported by allozyme data: 211 clones, the highest number ever reported for freshwater ostracods, have been determined in 1200 specimens from 55 European populations (Rossi *et al*., 1998). In the following paragraphs, we will discuss several hypotheses to explain such high genetic variability in a species with mixed reproduction. In particular, we will focus on multiple origins of asexuality, long persistence of asexual lineages and hybridization amongst sexual and asexual lineages.

Multiple origins of asexual reproduction

Neither of our data sets (ITS1 or COI) is consistent with a single origin of asexual reproduction in *E. virens*. This would have resulted in one asexual clade, whereas asexual individuals occur in at least four well supported clades in the ITS1 tree (Fig. 1) and the COI tree (Fig. 2). Assuming that sexual reproduction was the ancestral state on the basis of outgroup comparison and because reversal is expected to be difficult, a minimum of four independent origins of asexual reproduction can be inferred. We stress that this is a minimum estimate. The tree topology in Fig. 1 actually implies at least seven origins. In lineages like the one leading to the Berzosa, Morcuera and Pico samples (Fig. 1), the most parsimonious hypothesis is a single origin of asexuality before the first internal node within this clade. However, because it is clear that the evolution of asexual reproduction is not so rare as to be unique within this species, we cannot exclude the possibility that the lineage continued to reproduce sexually until a more recent date, which would imply a greater number of transitions to asexual reproduction.

Previous studies on asexual lineages in nonmarine ostracods (Turgeon & Hebert, 1995; Chaplin & Hebert, 1997) have implied a single origin for asexuality within each species studied, although many independent origins must have occurred in nonmarine ostracods as a whole (Chaplin *et al*., 1994). However, these studies have not included extant sexual populations from the screened species (which are, nevertheless, known in most of the species studied, e.g. *Heterocypris incongruens* — Martens, 1998), making the conclusion of a single origin inevitable. Multiple origins of clones from sexual ancestors could explain both the persistence of asexual reproduction and the extensive genetic diversity in *E. virens*.

Age of asexual lineages

The highest levels of divergence separate the Spanish asexual lineages of group II (Berzosa, Pico and Morcuera in Fig. 1) from both sexual populations and the other asexual lineages. They imply a maximum age for this group of about 4.5 Myr. These age estimates are considered upper limit estimates for the persistence of any asexual lineage amongst our samples. If a single origin of asexual reproduction is postulated, then the minimal persistence for asexuality in the lineage with the highest divergence (Group II, Fig. 1) is given by its deepest internal branching with about 2 Myr. However, if one allows the possibility of multiple origins, no minimum age can be specified. The data suggest that some origins of asexual reproduction are much more recent. For example, the asexual female from Ketton has a similar ITS1 sequence to individuals from the Sicilian sexual population.

Our mean estimates of 2.5–4.5 Myr for the common ancestry of ITS1 and COI amongst all the screened,

European *E. virens* populations suggest that most lineages diverged before the Pleistocene ice ages, with some clones representing Pliocene relicts. The oldest fossil record of *E. virens* in Spain is only 1 Myr (Anadon *et al.*, 1986), but as this species mainly inhabits temporary pools, fossil preservation of its large, fragile valves is rare. It is possible that large populations have persisted in southern Europe and north Africa throughout the Pleistocene glaciations and that central and northern Europe were recolonized after each glaciation by clones from refugial populations, which differed genetically to a large extent. They would have gradually expanded their range from the south to the north after each glaciation, as described for other groups such as grasshoppers (Cooper *et al.*, 1995). A post-Pleistocene range expansion could explain the present-day correlation between genetic and geographical distances and the surprisingly close relationships between the sexual population from Sicily and the asexual individuals from Belgium and England.

Our age estimates cannot prove that individual asexual clones of *E. virens* have persisted for several million years, because this would require a single origin of asexuality in this species. Our data, and considerations of the taxonomic distribution of parthenogenesis in nonmarine ostracods in general (Chaplin *et al.*, 1994), show that transitions from sexuality to asexuality are not rare. This makes it impossible to provide more than minimal estimates for the number of transitions between reproductive modes and maximal estimates for the ages of parthenogenetic lineages from molecular data. This is true, regardless of how well the phylogenetic hypotheses are supported and the level of confidence in the calibrations of divergence rates. Inclusion of more samples like another sexual population from northern Africa, would tend to increase the number of implied origins of asexual reproduction and decrease the apparent age of asexual lineages.

Congruence of phylogenetic hypotheses and hybridization

In strictly asexual lineages, mitochondrial and nuclear phylogenies should correspond perfectly. Comparison of phylogenies based on nuclear and mitochondrial sequence data can provide additional information on origin and persistence of asexual reproduction. Incongruence can be caused in two ways: asexual lineages may be recently derived from a large sexual population and contain random combinations of nuclear and mitochondrial variants from this ancestral gene pool, or asexual lineages are older and have accumulated their current levels of divergence over long independent histories but occasional hybridization between asexual females and sexual males has allowed exchange of genetic material. The latter possibility has been suggested in nonmarine ostracods as one source of polyploid clones with atypical allozyme genotypes (Turgeon & Hebert, 1995; Chaplin & Hebert, 1997).

There is evidence for incongruence between the ITS1 and COI phylogenies in *E. virens*, particularly in the placement of the two asexual females from Extremadura (Extr5 and Extr6) and for the Sanguinaro samples. The tree topologies for congruent data sets are not significantly different, because of the limited support for the ITS1 tree, and the placement of Extr5 and Extr6 is further complicated by three small indels. Nevertheless, we consider it most likely that the two exceptional females are the result of intraspecific hybridization between sexual and asexual lineages from Extremadura and are (at least) triploid. The grouping of the two asexual females (Ext5 and Ext6) with other asexual females from Pico in the ITS tree (Fig. 1) suggests that the same asexual lineages occur at Pico and Extremadura.

Our hypothesis is strongly supported by allozyme data of *E. virens* from the Extremadura site, which also provided evidence for intraspecific hybridization between asexual females and males (Rossi *et al.*, 1998). Courtship as well as copulation between males and asexual females of *E. virens* has been directly observed in the laboratory (Horne *et al.*, 1998). Furthermore, a high incidence of polyploidy has been demonstrated in *E. virens* females by chromosomal analysis (Tétart, 1978) and was (indirectly) inferred from allozyme data (Rossi *et al.*, 1998), but we do not know the ploidy levels of the sequenced individuals in the present study.

If the intraspecific hybridization occurred recently, the hybrids must be heterozygous for the ITS1 locus, assuming that the copies from the two parents differed. We found no evidence for heterozygosity from our direct sequencing, but the hybridization could have happened some time ago. In this case, concerted evolution of tandem-repeated genes like ITS will have reduced genetic variation through processes like gene conversion or unequal crossing-over. The latter homogenize genetically divergent repeats within individuals and have been found to continue operating in apomictic lineages (Crease & Lynch, 1991).

Genetic diversity in asexual lineages of *E. virens* is generated through three processes: the accumulation of mutations in extant clones, the continuous spin-off of new asexual lineages from sexual populations and mixed reproduction through occasional, intraspecific hybridization. All of them assure that in *E. virens* asexuality can persist in the long term.

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