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Genetic diversity and mixed reproduction in *Eucypris virens* (Crustacea: Ostracoda)

Valeria Rossi[1], Andrea Piotti[1], Angel Baltanás[2], Giorgio Benassi[1] and **Paolo Menozzi[1]**

With 5 figures and 3 tables

Abstract: We report data on the coexistence of apomictic and amphimictic lineages of *Eucypris virens* within the same pond and discuss the role of intraspecific hybridation as a source of genetic variability. We describe the genetic structure of 36 populations of *E. virens* from Europe. Most (28) populations were made up of putative parthenogenetic females, in 8 populations from Spain and Sicily we found males. Using three polymorphic allozyme markers (MPI, PGM and GPI) we described at least 214 different multilocus genotypes (clones) in a total of 1151 analysed specimens and confirmed that parthenogenetic reproduction is apomictic. Most multilocus genotypes, 74 %, were limited to a single population indicating the absence of a single widespread 'clone'. In *E. virens*, genetic diversity was not affected by latitudinal cline or reproductive mode and was probably the result of several processes. Apomictic lineages were the likely result of at least two independent transitions from different sexual ancestors located in Southern Europe, in Central Spain and in Sicily. Transitions probably happened after the last glaciation. We found sympatric amphimictic and apomictic females in Extremadura (Western Spain) and reported evidence of gene flow between different lineages (intraspecific hybridation). Further genetic differentiation within and among populations reflects time of divergence and mutation accumulation. We found putative polyploid multilocus genotypes in all populations with males, further support for the coexistence of lineages with different reproductive modes. The presence of polyploid genotypes (14 %) is probably underestimated and this limits our ability to assess the role of hybridisation as a major route to parthenogenesis.

Key words: geographic parthenogenesis, allozyme markers, clonal diversity, hybridisation, ephemeral habitats.

Introduction

The genetic variability of parthenogenetic species is well known and has been indicated as an explanation for the widespread distribution and the persistence of organisms that, for decades, were considered an evolutionary dead end (Bell 1982). The source of genetic variability is often linked to the origin of parthenogenesis: mutation, autopolyploidisation, intraspecific and interspecific hybridisation, spontaneous or contagious loss of sex (Simon et al. 2003). Selection by environmental factors and interaction between genotype and environment in morphology, tolerance and competitive ability, have also been used to explain the distribution and the maintenance of clonal variability (Hughes 1992, Wilson & Hebert 1992, De Meester 1996, Weider et al. 2005). The "frozen niche variation" hypothesis sees parthenogenetic species as made up of an array of clones (multilocus genotypes), each with a unique, relatively narrow niche, representing a particular combination of fitness-related life history traits (Vrijenhoek 1978, 1984). The "frozen niche variation" model predicts that local abundance and frequency of asexual lineages is positively correlated to the amount of

[1] **Authors' addresses:** Department of Environmental Sciences, University of Parma, Viale G.P. Usberti 11/A, 43100 Parma, Italy.
[2] Department of Ecology, Autónoma University of Madrid, c/Darwin 2, 28049 Madrid, Spain.
***** Corresponding author; e-mail: valeria.rossi@unipr.it

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clonal diversity. Species with mixed breeding systems would have the opportunity for repeated transitions to asexuality and for recruitment of new genotypes from sexual relatives (Bell 1982). Geographic parthenogens are a special case of mixed breeding systems where sexual (organisms reproducing with fertilization and meiosis (mixis)) and asexual (organisms reproducing without fertilization) populations of a single species have, by definition, a disjointed spatial distribution (Bell 1982, Suomalainen et al. 1987). Many parthenogens show broader geographic distributions than their sexual relatives (Lynch 1984).

Ostracods are one of the largest groups of Crustacea and have been reported as being among the most genetically variable parthenogens (Havel & Hebert 1993, Turgeon & Hebert 1995, Cywinska & Hebert 2002, Little 2005, Rossi et al. 2006). Gender is chromosomally determined and, generally, the male kariotype shows a variable number of heterochromosomes without homologous (Schön & Martens 1998). A wide geographical dispersal has been reported for clonal lineages (Chaplin & Hebert 1997, Chaplin & Ayre 1997, Little & Hebert 1997). In non-marine species, the high incidence of transition from sexual to asexual reproduction probably generates the bulk of "frozen" genetic diversity from sexual to asexual lineages (Havel et al. 1990ab, Chaplin et al. 1994). Turgeon & Hebert (1994) suggest that both the incidence of spontaneous transition to clonality (asexual reproduction without meiosis, synonym of apomixis) and the interbreeding with sexual congeners or relatives may be the processes that govern clonal diversity in asexual ostracods. In *Cypris pubera,* Little (2005) found a linkage between genetic diversity and polyploidy that might have arisen through internal duplication or hybridisation. In a sample of 286 ostracod species, 57 % show coexisting sexual and asexual lineages (Butlin et al. 1998), but very few species with mixed reproductive mode were studied and, above all, few comparisons among populations with different breeding systems were published (Chaplin & Ayre 1989). Chaplin (1992) investigated the relationship between sexual and asexual populations of *Candonocypris novaezelandiae* demonstrating the coexistence of sexual and asexual females and the local displacement of sexually reproducing lineages by conspecific parthenogenetic ones (Chaplin 1993). We described coexisting sexual and asexual populations of individuals belonging to different species of the genus *Heterocypris*: *H. barbara* and *H. incongruens* (Rossi et al. 2007). Apomictic lineages of *Heterocypris* are a result of independent transitions to apomixis from different sexual ancestors and time of divergence reflected the genetic differentiation within and among them.

Eucypris virens is a cosmopolitan species, widely distributed in North Africa, Europe and Central Asia, typical of winter-early spring temporary ponds. In Europe the species shows geographic parthenogenesis while it is reported as an obligate parthenogen in North America (Bell 1982, Horne et al. 1998). Asexual populations are found in much of Europe and sexual populations have been described in the southern part of the species' biogeographic range where both parthenogenetic and sexual populations were found in restricted areas (Horne et al. 1998). This distribution fits with the "Pleistocene hypothesis", that explains the presence of sexual populations in the South as remnants of populations that survived in ancient refugial areas and parthenogenetic populations in the North as a result of postglacial recolonisation. *E. virens* is a swimming species of cold water habitats (Geiger et al. 1998). Its univoltine life cycle might account for the generally low population densities and for the reduced thermal niche differentiation. Laboratory experiments indicate that voltinism may depend on the latitudinal adaptation of different clones (Otero et al. 1998).

Here we report a large scale study of genetic variability of sexual and asexual European *E. virens* populations. DNA sequence data, used in a previous phylogenetic reconstruction, revealed multiple independent origins of main clonal lineages from most of these populations and, for some of them, long term parthenogenesis (Schön et al. 2000). Using allozyme markers, whose resolving power was previously described (Rossi et al. 1998), we evaluate the species multilocus genotype diversity and discuss the possible gene flow between sexual and asexual lineages and its role as a source of genetic variability.

Methods

More than 200 temporary habitats were visited in different European localities and *E. virens* populations were sampled in 36 water bodies from Great Britain (1 site, code 1), Belgium (1 site, code 2), Italy (22 sites, codes 3–17 and 30–36) and Spain (12 sites, codes 18–29) (Table 1). Sampling in Italy was performed in rice fields located in the Po plain (codes 3–9) (Rossi et al. 2006) and in temporary ponds from Northern Italy (codes 10–14), Northern Apennines (code 15), Central Italy (code 17), an island off the Tuscan coast (code 16) and Sicily (codes 30–36). Sampling in Sicily was performed inland in order to find temporary ponds where Crosetti & Margaritora (1982) found males of *E. virens* in the nineteenseventies. Samples from Spain came from ponds located in Central (codes 18–20) and Western Spain

(Extremadura) (codes 21–29) from an area of about 900 km^2. In this region the sexual populations and asexual populations co-occur inhabiting nearby ponds.

Qualitative samples were collected with a net (25 cm diameter, 20 µm mesh). Live specimens were sorted and checked for species and gender by using a Wild M8 stereomicroscope and analysed within 4 days from sampling or frozen at –80 °C until analysis.

For testing apomictic parthenogenesis and ensuring consistency in allelic designation of electrophoretic enzymatic patterns, laboratory clonal lineages were obtained by isolating single immature females, randomly selected from different populations. Each clonal lineage was maintained in test tubes with 20 ml of Evian spring water, at 16°C (12:12 L:D photoperiod) and fed *Tolypothrix tenuis* (Cyanobacteria). Electrophoretic analysis was repeated in different clonal lineages for at least 3 generations, even in individuals from resting eggs. Females from tested clonal lineages were used as internal standards on all electrophoretic analysis.

The genetic structure of *E. virens* populations was determined by screening for variation at eight enzymes loci: GOT (glutammate-oxaloacetate transaminase E.C.2.6.1.1), GPI (phosphoglucose isomerase E.C.5.3.1.9), IDH (isocitrate dehydrogenase E.C.1.1.1.42), MDH (malate deydrogenase E.C.1.1.1.37), ME (malic enzyme E.C.1.1.1.40), MPI (mannose phosphate isomerase E.C.5.3.1.8), 6PGD (phosphogluconate dehydrogenase E.C.1.1.1.44), PGM (phosphoglucomutase E.C.2.7.5.1). Each individual homogenate was diluted in 40 µl of distilled water and analysed by horizontal starch gel (12 %) electrophoresis. Each allele is identified by its relative anodal migration according to increasing alphabetic order. The presence of supernumerary bands was considered evidence of polyploidy (Little & Hebert 1994). The combined genotypes of the polymorphic loci were used to define the multilocus genotype (MLG) of each individual.

Number of alleles per locus, number of MLGs, number of putative polyploidy genotypes and genotype diversity index (G/N) were calculated from the scores obtained from individual organisms for each population. The genotype diversity index was calculated as the ratio between the number of different MLG and the sample size. This index ranges from zero, when all individuals are copies of a single genotype (apomictic reproduction), to 1, when all individuals have distinct genotypes (amphimictic reproduction). The analysis of genetic diversity was carried out using the software GenoType/GenoDive (Meirmans & Van Tienderen 2004). Nei's genetic diversity corrected for sample size was calculated for each population and a jackknife procedure with 1000 permutations was used to check sample size adequacy in diversity index estimation. Pairwise distances between MLGs were calculated assuming an infinite allele model. Allele frequencies and heterozygosity were evaluated in populations of more than 20 individuals and with more than one MLG. In these cases percentage of polymorphic loci, mean heterozygosity by direct count, fixation index (F_{IS}) (Weir & Cockerham 1984) and global linkage disequilibrium between pairs of loci were computed using the GENEPOP package version 3.4 (Raymond & Rousset 1995). A Markov chain method was used to estimate the P-value of deviations from equilibrium in the Hardy-Weinberg exact test (Guo & Thompson 1992). To evaluate the distribution of genetic diversity among and within populations the analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was computed with GenoType/GenoDive (Meirmans & Van Tienderen 2004) in order to account for polyploidy individuals, otherwise excluded from the analysis. Genetic differentiation was also evaluated using Nei's genetic similarity index (Nei 1978).

Two different approaches were used to describe the relationship between genetic differentiation and geographic distribution. A Principal Component Analysis for categorical data using the statistical package SPSS® version 14.1 was performed to distinguish and discriminate populations according to the presence or absence of different alleles at polymorphic loci. The relationship between the two first principal components and geographic position was investigated using multiple regression. Furthermore, two dimensional Principal Coordinate Analysis was performed on the inter-genotype distance matrix based on the Manhattan distance computed with Genotype/Genodive (Meirmans & Van Tienderen 2004), using the function "cmdscale" of the R statistical package (R Development Core Team 2004). In addition, the phylogeography of genotypes was assessed by spatial autocorrelation using the multilocus genetic similarity index $r^{(h)}$, based on the procedure described in Smouse & Peakall (1999) and calculated by the software GenAlEx (Peakall & Smouse 2005). Statistical significance of $r^{(h)}$ values was evaluated calculating the 95 % confidence interval under the null hypothesis of no genetic structure (999 permutations). The relationship between genetic and geographic distance was also estimated using a Mantel test (R statistical package).

An assignment test, to calculate the probability that an MLG may originate from a population other than the one it was found in and to evaluate the presence of admixed individuals, was carried out using a Bayesian clustering approach (Pritchard et al. 2000). This method was implemented by a Markov chain Monte Carlo algorithm that allows the estimate of the relative contribution (q) of K predefined clusters to each individual multilocus genotype. Parameters estimation assumes Hardy-Weinberg equilibrium within populations and linkage equilibrium, but the Bayesian approach is robust to same deviations from this assumption (Falush et al. 2003, Halkett et al. 2005). The results presented were obtained using an admixture model and are based on a Markov chain with 5×10^5 iterations, following a burning period of 10^5 iterations, using the software STRUCTURE version 2.2 (Pritchard et al. 2000).

Results

A total of 1151 individuals were screened in 36 temporary ponds (Table 1). In 28 populations only females were found (hereafter unisexual populations) while in 2 populations from Spain and in 6 populations from Italian temporary ponds located in Sicily males and females were recorded (Table 1). In sexual populations sex ratio varied between 0.06 and 1.22.

MDH locus was monomorphic, while ICD1, ICD2, ME, MPI, PGM, GPI, 6PGD, and GOT loci were polymorphic. Several individuals in different populations failed to show any activity or clear polymorphism at several of these loci. For this reason, only MPI, GPI and PGM loci were considered for further analysis. The combined genotypes of these three loci defined the multilocus genotype (MLG) of each individual. A

Table 1. For each locality (country) latitude, longitude, sample size (N), number of alleles per population at three polymorphic loci (MPI, PGM and GPI) (All), number of multilocus genotypes (MLG), percentage of putative polyploid genotypes (PPG), Nei's genetic diversity index corrected for sample size (Div), genotype diversity index (G/N) and sex ratio (M/F) are reported. Only one and two loci were scored at Randazzo and Cabriolo, respectively.

Population	LAT	LON	N	All	MLG	PPG	Div	G/N	M/F
1 Bramhope (GB)	53° 50′ N	01° 35′ W	37	5	1	0	0.000	0.03	0
2 Haantjen (B)	51° 06′ N	03° 38′ E	8	12	4	25	0.750	0.50	0
3 Briona (I)	45° 32′ N	08° 28′ E	25	11	6	0	0.533	0.24	0
4 Cameri (I)	45° 30′ N	08° 39′ E	4	8	3	0	0.833	0.75	0
5 Gionzana (I)	45° 26′ N	08° 32′ E	3	7	2	0	0.667	0.67	0
6 Murone (I)	45° 16′ N	08° 05′ E	43	8	4	0	0.258	0.09	0
7 Saluggia (I)	45° 14′ N	08° 00′ E	22	7	6	0	0.645	0.27	0
8 Torrebianca (I)	45° 11′ N	09° 07′ E	4	6	3	0	0.833	0.75	0
9 La Bigliana (I)	44° 51′ N	10° 36′ E	31	12	16	6	0.915	0.52	0
10 Colorno (I)	44° 55′ N	10° 22′ E	130	20	40	22	0.805	0.31	0
11 Torrile (I)	44° 55′ N	10° 19′ E	65	15	16	25	0.910	0.25	0
12 Sanguinaro (I)	44° 47′ N	10° 10′ E	32	13	12	0	0.843	0.38	0
13 Cabriolo (I)	44° 50′ N	09° 48′ E	5	5	3	0	0.700	0.60	0
14 Piallassa (I)	44° 28′ N	12° 17′ E	9	6	2	50	0.389	0.22	0
15 Code d'Asino (I)	44° 29′ N	09° 28′ E	78	6	1	0	0.000	0.01	0
16 Capraia (I)	43° 02′ N	09° 48′ E	2	4	1	0	0.000	0.50	0
17 Ventina (I)	42° 30′ N	12° 40′ E	10	8	5	0	0.800	0.50	0
18 Morcuera (E)	40° 50′ N	03° 50′ W	71	4	1	0	0.000	0.01	0
19 Berzosa (E)	40° 36′ N	03° 56′ W	28	12	11	0	0.820	0.39	0
20 Canto del Pico (E)	40° 35′ N	03° 55′ W	70	8	10	0	0.629	0.14	0
21 Extremadura (E)	39° 31′ N	06° 15′ W	27	9	8	0	0.786	0.30	0
22 Extremadura 1 (E)	39° 55′ N	06° 04′ W	20	11	11	0	0.889	0.55	1.22
23 Extremadura 4 (E)	39° 41′ N	05° 58′ W	14	11	8	13	0.972	0.57	0
24 Extremadura 5 (E)	39° 39′ N	06° 17′ W	15	11	7	0	0.781	0.47	0
25 Extremadura 9 (E)	39° 28′ N	06° 09′ W	13	10	10	20	0.949	0.77	0
26 Extremadura 10 (E)	39° 35′ N	06° 05′ W	12	6	3	0	0.621	0.25	0
27 Extremadura 12 (E)	39° 44′ N	06° 05′ W	18	12	9	11	0.856	0.50	0
28 Extremadura 16 (E)	39° 54′ N	06° 03′ W	69	13	19	5	0.907	0.28	0.21
29 Extremadura 18 (E)	39° 56′ N	06° 04′ W	9	8	4	25	0.694	0.44	0
30 Randazzo – Sicily (I)	37° 51′ N	14° 51′ E	31	2	1	100	0.000	0.03	0
31 Mistretta 7 – Sicily (I)	37° 49′ N	14° 24′ E	9	6	4	0	0.806	0.44	0.50
32 Mistretta 9 – Sicily (I)	37° 49′ N	14° 24′ E	34	8	19	5	0.948	0.56	0.42
33 Mistretta 10 – Sicily (I)	37° 49′ N	14° 24′ E	86	7	28	14	0.953	0.33	0.23
34 Mistretta 12 – Sicily (I)	37° 49′ N	14° 24′ E	18	7	13	15	0.967	0.72	0.39
35 Mistretta 13 – Sicily (I)	37° 52′ N	14° 23′ E	50	8	15	0	0.895	0.30	0.06
36 Nicosia 37B – Sicily (I)	37° 41′ N	14° 23′ E	49	4	3	0	0.166	0.06	0.40

total of 12, 11, and 5 alleles were detected at MPI, GPI and PGM loci, respectively. The number of alleles per population significantly increased with sample size in unisexual populations ($R^2 = 0.176$, $F = 5.122$, $P = 0.033$) although the relationship was affected by a large dispersion. The mean number of alleles per locus was not different between unisexual (mean = 9.19, SD = 3.666) and sexual populations (mean = 8.00, SD = 2.828) ($P = 0.406$). Most alleles were found in most populations. At MPI locus, one allele was private of the Ventina population, and two alleles were private for the unisexual Spanish populations from Berzosa and Extremadura region. At GPI locus two alleles were private of the English genotype from Bramhope and of the Spanish population from Berzosa, respectively. A third allele was limited to Spanish populations from Canto del Pico and Extremadura region. Most populations from the same restricted geographic area were grouped on the basis of shared alleles (Fig. 1). The percentage of explained variance was 20 % and 14 % for the first and second component, respectively. Sexual populations from Sicily (Nos 31–36) were well separated from both Spanish sexual populations (Nos 22 and 28) and all unisexual populations except the

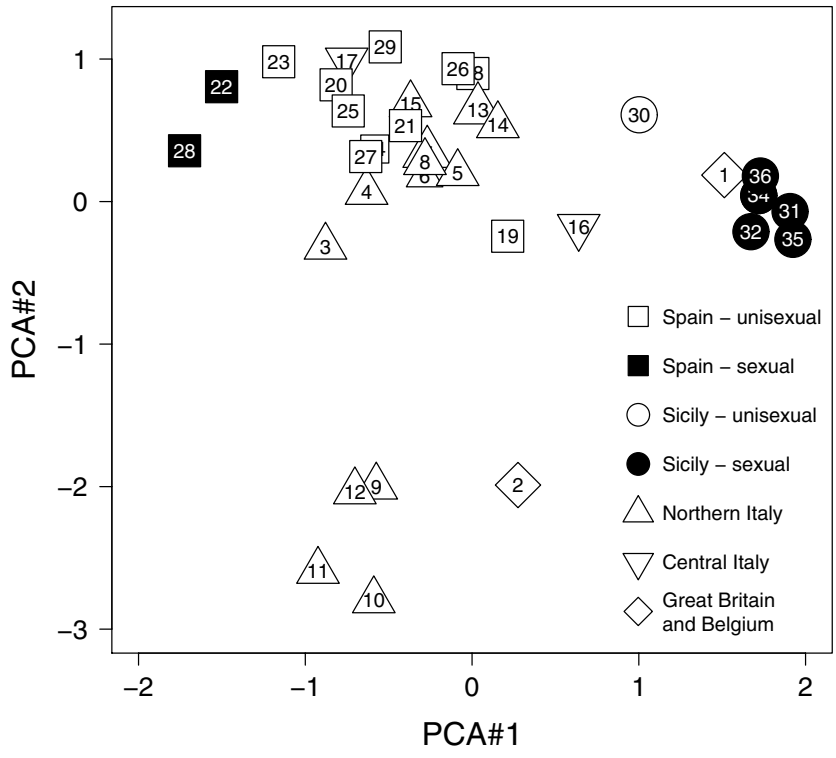

Fig. 1. Scatterplot of population scores by Principal Component Analysis for Categorical Data according to the presence or absence of different alleles at polymorphic loci. See Table 1 for population codes.

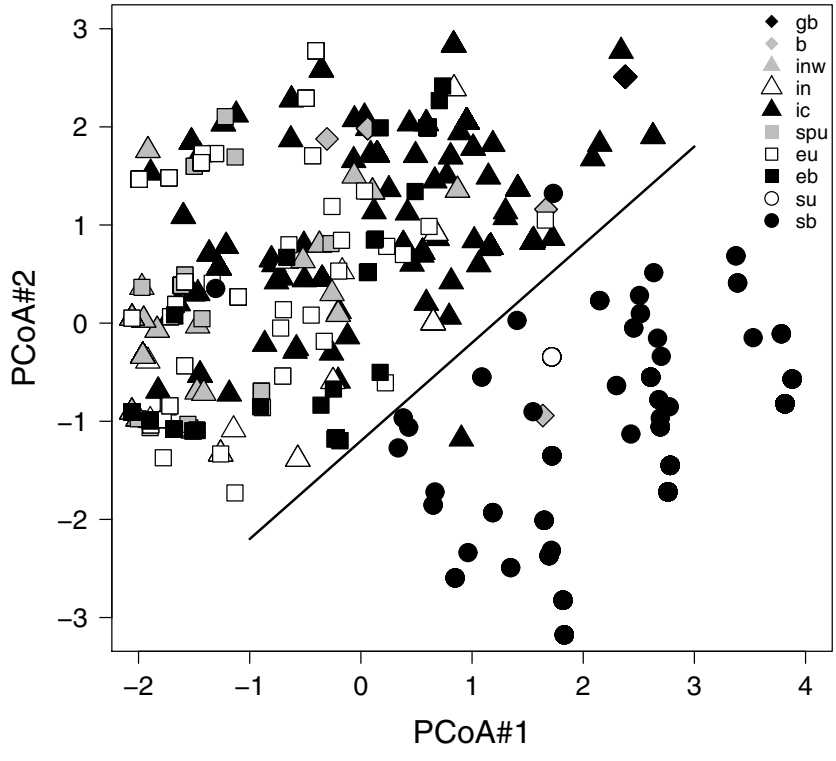

Fig. 2. Scatterplot of MLG score by Principal Coordinate Analysis according to their genetic similarity. Black and grey lozenges indicate MLGs from British and Belgian populations, respectively. Grey, white and black triangles indicate MLGs from unisexual populations from North-Western (3–8), Northern (9–15), and Central (16–17) Italian populations, respectively. White, black and grey squares indicate MLGs from sexual and unisexual Extremadura (Western Spain) populations and unisexual populations from Central Spain (18–20), respectively. White and black circles indicate MLGs from sexual and unisexual Sicilian populations, respectively. See Table 1 for population codes.

monoclonal one from Great Britain (No 1). Four Italian populations (Nos 9–12), all located in a relatively restricted area in Northern Italy (about 500 km^2), shared one allele at MPI locus with Spanish sexual populations from Extremadura and had, as well as the Belgian population from Haantjen (No 2), a private allele at the GPI locus. All other Italian populations were associated according to their geographic locali-

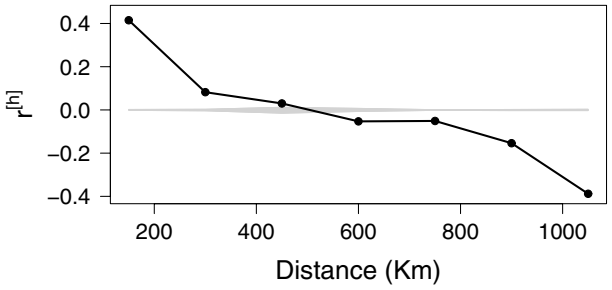

Fig. 3. Correlogram of the genetic correlation coefficient $r^{(h)}$ as a function of geographic distance. Shaded area indicates the 95 % confidence interval of the null hypothesis, calculated with 999 permutations.

sation. In general, the distribution of each population was well explained by its geographic position; in fact PC1 scores depended both on latitude and longitude and on their interaction (Multiple $R^2 = 0.8207$, $F_{3,32}$, $P < 0.001$).

A total of 214 different MLGs were detected. Most populations hosted more than one MLG with the exception of Bramhope, Code d'Asino, Morcuera, Randazzo (Table 1). Most MLGs, 74 %, are limited to single populations. At all loci, in heterozygotes, unbalanced staining bands were found. This fact suggested the existence of extra-gene copies, ascribable to polyploidy, in different heterozygous genotypes from different populations. Thirty multilocus genotypes (14 %) show unbalanced staining in at least one locus (Table 1). The Randazzo population was exclusively made up of such genotypes. Polyploid genotypes were present both in unisexual and sexual populations from Extremadura and Sicily and their abundance was not related to a latitudinal cline. The number of MLG significantly increased with sample size in unisexual populations ($R^2 = 0.413$, $F = 18.283$, $P < 0.001$). In unisexual populations the number of MLG and the genetic diversity index were positively related to the number of alleles ($R^2 = 0.759$, $F = 75.383$, $P < 0.001$ and $R^2 = 0.415$, $F = 17.057$, $P < 0.001$, respectively). Mean number of MLGs per population was significantly higher in sexual populations (mean = 14.0, SD = 8.264) than in asexual populations (mean = 7.04, SD = 7.801) ($P = 0.035$) while the mean genetic diversity index was not different between sexual (mean = 0.82, SD = 0.268) and unisexual populations (mean = 0.61, SD = 0.328) ($P = 0.108$). The distribution of all pairwise genetic distances between MLGs was skewed to the left. MLGs were sorted into two main distinct groups (Fig. 2). One group was made up of MLGs from Sicilian sexual and unisexual populations and included one MLG from the Belgian unisexual population (mostly below the line in Fig. 2). The other group was principally made up of MLGs from Spanish sexual and unisexual populations, Italian unisexual populations and included MLGs from the Belgian population (mostly above the line in Fig. 2). MLG from the Great Britain clustered with the first group

Table 2. For polymorphic populations with N > 20, mean numbers of alleles per locus, mean heterozygosity by direct count (H DC) and mean heterozygosity expected according to Hardy-Weinberg equilibrium (H HW) are reported with standard deviation in parentheses. Fixation index (F_{IS}) for significant heterozygote deficiency ($F_{IS} > 0$) or excess ($F_{IS} < 0$) for each polymorphic locus is reported; ns indicates that the locus is in Hardy-Weinberg equilibrium. In all populations, all loci are polymorphic at the 0.99 criterion with the exception of Nicosia 37B.

	N	alleles	H DC	H HW	MPI	PGM	GPI
Briona	25	3.2 (1.2)	.883 (.075)	.603 (.077)	–.382	–.280	–.789
Murone	43	3.0 (0.6)	.915 (.048)	.509 (.010)	–.868	–.662	–.867
Saluggia	22	6.0 (0.6)	.581 (.426)	.371 (.247)	–.590	–.636	ns
La Bigliana	22	2.8 (1.0)	.603 (.033)	.645 (.133)	.174	ns	ns
Colorno	91	3.3 (1.5)	.890 (.064)	.694 (.038)	–.306	–.277	–.263
Torrile	49	4.3 (1.2)	.817 (.100)	.687 (.056)	–.334	–.176	.065
Sanguinaro	32	4.2 (1.0)	.600 (.441)	.432 (.298)	–.389	–.440	–.151
Berzosa	28	3.2 (1.7)	.417 (.324)	.466 (.266)	–.155	.480	ns
Canto del Pico	70	2.2 (1.0)	.338 (.256)	.274 (.191)	–.354	ns	ns
Extremadura	27	3.0 (1.5)	.471 (.433)	.492 (.360)	.217	–.243	ns
Extremadura 1	20	3.0 (1.5)	.110 (.069)	.411 (.101)	.535	.712	ns
Extremadura 16	66	2.9 (1.5)	.447 (.241)	.577 (.139)	.241	.584	–.045
Mistretta 9	32	2.6 (0.6)	.384 (.153)	.446 (.078)	ns	ns	ns
Mistretta 10	69	2.6 (0.6)	.393 (.177)	.444 (.089)	.386	ns	ns
Mistretta 13	50	2.7 (0.6)	.373 (.202)	.356 (.198)	ns	ns	ns
Nicosia 37B	49	3.1 (0.6)	.043 (.028)	.029 (.025)	ns	ns	ns

according to axis 1 and with the second group according to axis 2. Both groups were well scattered in a two dimensional Euclidean space confirming a high similar intra-group genetic variability. Spatial autocorrelation analysis revealed significant spatial MLG structure up to relatively long distances (~ 450 km) (Fig. 3) and the geographic structure is confirmed by the result of Mantel test, that showed a statistically significant relationship between genetic and geographic distances (999 re-sampling cycles, $R_{xy} = 0.521$, $P = 0.01$).

In polymorphic populations with sample size larger than 20 individuals, a significant departure from Hardy-Weinberg equilibrium was observed in unisexual populations with the exception of Saluggia, Berzosa and Extremadura at GPI locus, La Bigliana and Canto del Pico at both the PGM and GPI loci (Table 2). In most cases disequilibrium was due to heterozygote excess. On the other hand, all sexual populations were in Hardy-Weinberg equilibrium with the exception of Extremadura16 at all loci, Extremadura1 at MPI and PGM loci and Mistretta10 at MPI locus. In most cases disequilibrium was due to heterozygote deficit. Mean heterozygosity is higher in unisexual (0.652) than in sexual (0.289) populations ($F = 12.416$, $P = 0.003$). All loci pairs were at significant linkage disequilibrium in the Briona, Murone, Colorno, Torrile, Sanguinaro and Extremadura16 populations; MPI-PGM and MPI-GPI pairs were at significant linkage disequilibrium in the La Bigliana and Berzosa populations, MPI-PGM pair was at significant linkage disequilibrium in the Saluggia, Canto del Pico, Extremadura, Mistretta9 and Mistretta10 populations. Among all populations genetic differentiation was significant (AMOVA phi-statistics = 0.039, $P = 0.001$) as well as among all unisexual (AMOVA phi-statistics = 0.049, $P = 0.001$) and among all sexual (AMOVA phi-statistics = 0.098, $P = 0.001$) ones.

Table 3. Proportion of specimens of each population from Extremadura assigned to the 4 clusters by STRUCTURE with probability equal to or higher than 90 %. Proportion of unassigned specimens (Un) and sample size (N) are also reported.

	Inferred Clusters					
Population	1	2	3	4	Un	N
21 Extremadura	0.48	0.00	0.19	0.00	0.33	27
22 Extremadura 1	0.00	0.10	0.00	0.85	0.05	20
23 Extremadura 4	0.25	0.00	0.13	0.00	0.62	8
24 Extremadura 5	0.07	0.00	0.60	0.00	0.33	15
25 Extremadura 9	0.27	0.36	0.00	0.00	0.36	11
26 Extremadura 10	0.00	0.00	0.83	0.00	0.17	12
27 Extremadura 12	0.00	0.00	0.50	0.00	0.50	12
28 Extremadura 16	0.00	0.49	0.00	0.44	0.07	68
29 Extremadura 18	0.00	0.14	0.00	0.00	0.86	7

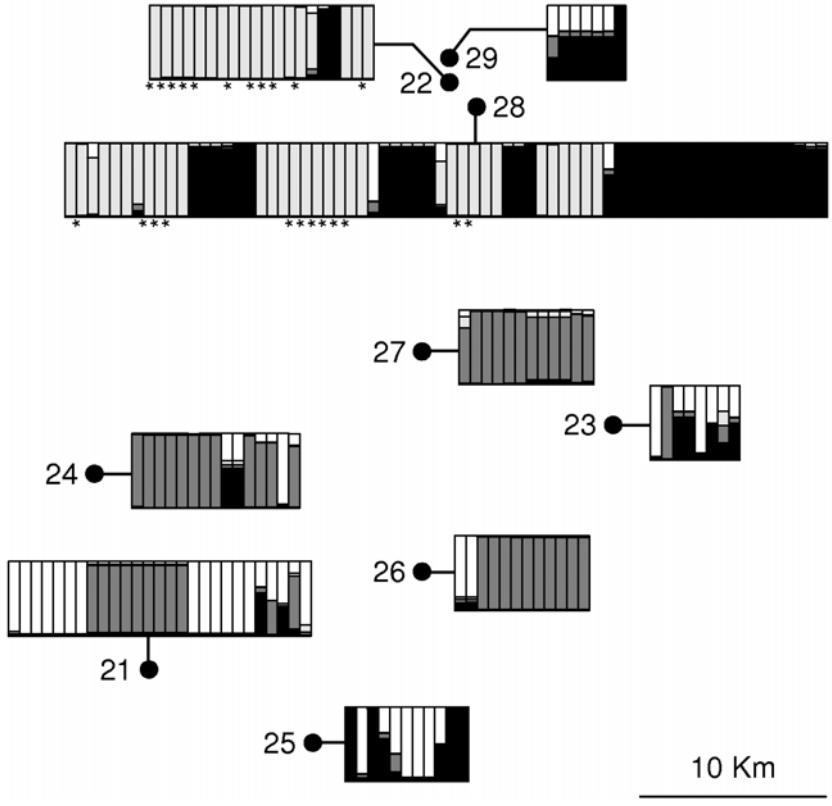

Fig. 4. Geographic distribution of the nine Extremadura populations (each number indicates a population, see code in Table 1). For each population a barplot indicates the estimated genetic contribution of the four inferred groups into the individual genotypes: cluster 1 (white), cluster 2 (black), cluster 3 (dark grey) and cluster 4 (light grey). Each bar indicates an individual (see Table 3) and asterisks indicate male individuals.

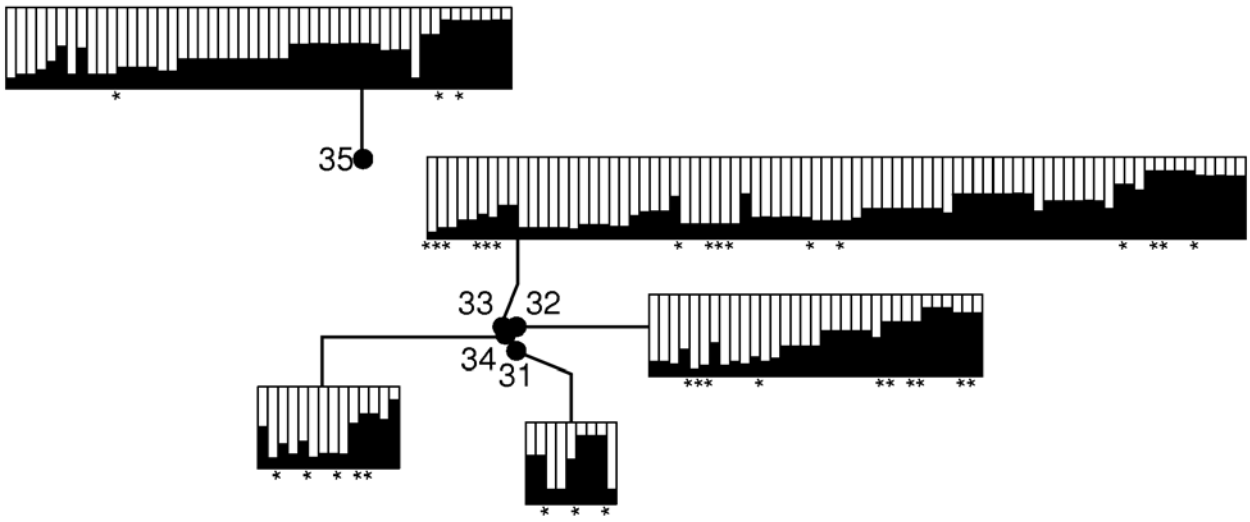

Fig. 5. Geographic distribution of six Sicilian populations (all except Randazzo) (each number indicates population, see code in Table 1). For each population position, a barplot indicates the estimated genetic contribution of the 2 inferred groups into the individual genotypes: cluster 1 (black), cluster 2 (white). Each bar indicates and individual and asterisks indicate male individuals.

Two assignment tests were performed, separately, on the polymorphic populations from Extremadura and Sicily. In the Extremadura populations, the *a posteriori* allele frequency distribution was best fitted assigning individuals to four clusters (K = 4, matching probability ~ 1). In general, assignment probabilities were high especially in the sexual populations of Extremadura 1 and Extremadura 16 and 76 % of all analysed specimens were assigned with a probability higher than 90 % (Table 3). Low assignment probabilities were observed in very small populations with sample size less than ten. Cluster 4 included all males (23) and 67 % and 33 % of females, from sexual populations of Extremadura 1 and Extremadura 16, respectively. Among the females from these sexual populations 22 % and 58 %, respectively, were assigned to cluster 2 as well as 36 % and 14 % of putative apomictic females from unisexual populations of Extremadura 9 and Extremadura 18, respectively. Most of the putative apomictic females from unisexual populations were assigned to clusters 1 (27 %) and 3 (27 %) (Fig. 4). In the group of males and putative amphimictic females from Extremadura 16 and Extremadura 1 that were assigned to cluster 4, genotype frequencies were both in Hardy-Weinberg and linkage equilibrium. On the other hand, heterozygote excess at MPI (F_{IS} = –0.486) and GPI (F_{IS} = –0.620) loci and linkage disequilibrium were detected in the group of females from Extremadura 16 that were assigned, with putative apomictic females from unisexual populations, to cluster 2. Nei's genetic similarity index between females from Extremadura 16 that grouped in cluster 4 and in cluster 2 was lower than 50 % and the amount of genetic differentiation was relatively high (F_{ST} = 0.281) (AMOVA phi-statistics = 0.082 P = 0.0160). In populations from Sicily, the *a posteriori* allele frequency distribution was best fitted assigning individuals to two clusters (K = 2, matching probability ~ 1). Assignment probabilities were very low: only 0.8 % of all analysed specimens were assigned with a probability higher than 90 % (Fig. 5). Decreasing probability threshold to 80 %, most specimens from Nicosia (92 %) were assigned to cluster 1 while 23 % of specimens from Mistretta area were assigned to cluster 2. Many of the unassigned females had a recent ancestry in the Nicosia population. Nei's genetic similarity between these two main groups is 90 %.

To test apomictic parthenogenesis, 170 laboratory clones from 15 all-female populations, were analysed: 12 % of clones were homozygous at 3 markers, 21 % of clones were heterozygous at 1 locus, 31 % at 2 loci, 28 % at 3 loci and 8 % contained at least one polyploid locus. All clonal populations were made up of females. Only due to the univoltinism of many clones most eggs were diapausal. For this reason relatively few females per generation per clones were tested. Nevertheless recombination was totally absent in 353 specimens from 207 different generations and unbalanced staining electrophoretic phenotypes is maintained in polyploid clones.

Discussion

Our data confirm a very high level of genotypic diversity in *E. virens*, in line with previous data on apomictic species. Comparative studies revealed an even higher level of clonal diversity in genera containing sexual species or geographic parthenogentic ones (Bell 1982, Cohen & Morin 1990). In *Cypridopsis vidua* over 200 different clones were identified in a survey of 47 populations in North America (Havel & Hebert 1993, Cywinska & Hebert 2002). Havel et al. (1990b) described 66 different clones among 1023 specimens of *Cypricercus reticulatus* from 20 habitats in a relatively limited low Arctic region near Churchill and, for the same species, Turgeon & Hebert (1995) reported 185 different clones in a survey of 31 ponds from the Laurentian Great Lakes area. In Northern Italy (Po plain) up to 125 different clones of *Heterocypris incongruens* were found (Rossi et al. 2006). We found that in unisexual populations of *E. virens* the number of MLGs was significantly related to allele number, that is to the accumulation of "new alleles" created by mutation and maintained in absence of recombination (apomixis). Many authors stressed the general validity of breeding system assignment, through both sex ratio and genotypic frequency approaches (Havel & Hebert 1989, Chaplin 1993). What we observed, the presence of males, Hardy-Weinberg equilibrium and the lack of genome wide linkage disequilibrium can be considered evidence of sexual reproduction. Apomixis, that is considered the mode of reproduction in parthenogentic unisexual populations, is generally associated with heterozygote excess and linkage disequilibrium. In geographic parthenogen *E. virens*, genetic diversity was not affected by latitudinal cline or reproductive mode and was probably the result of several synergic processes such as transition from ancestral sexual populations, mutation accumulation, hybridisation and polyploidy (Turgeon & Hebert 1994, Chaplin et al. 1994, Turgeon & Hebert 1995, Little & Hebert 1997, Schön et al. 2000, Cywinska & Hebert 2002, Little 2005).

The two sexual population groups from Spain and Sicily share more alleles and multilocus genotypes, even polyploid ones, with unisexual populations from their geographic area than with each other. The allele and MGL distributions suggested that most apomictic lineages might be the result of at least two independent transitions from different sexual ancestors located in Southern Europe (in Western Spain and in Sicily). Our results, in accordance with molecular data (Schön et al. 2000) suggest a multiple origin of parthenogenesis in *E. virens*. According to the Pleistocene hypothesis, parthenogenetic lineages were widespread in the Northern regions that were recolonised after the last Ice Age (Horne et al. 1998). Most MLGs were more or less recent descendants from Spanish populations. MLGs derived from Sicilian sexual lineages, that might have been introduced from Northern Africa (Gauthier 1928, Masi 1932, Klie 1943), were less frequent: among them, the MLG from Great Britain and some Belgian ones (see above). Spatial autocorrelation analysis revealed significant spatial MLG structure up to relatively long distances (~ 450 km) that might be a mean geographic distance between sources of variability. Transition from sexual lineages happened more than once since the last glaciation and most of the initial variability was probably retained after fixation of genotype variation by apomixis. The putative influence of recent transition resulted from Extremadura data. As expected, genotype frequencies were in Hardy-Weinberg and in linkage equilibrium in sexual populations but significant disequilibrium was observed in Extremadura 1 and Extremadura 16 sexual populations. According to assignment test, two females from Extremadura 1 and 33 females from Extremadura 16 sexual populations did not cluster with males but with putative apomictic females from nearby unisexual populations. This result should indicate the coexistence in Extremadura 1 and Extremadura 16 of apomictic and amphimictic females and that genetic flow between amphimictic and apomictic lineages (intraspecific hybridisation) is currently happening or has taken place in very recent time. This result represents a significant support to the hypothesis of hybridization between Extremadura males and parthenogenetic females formulated by the comparative analysis of mitochondrial and nuclear DNA sequences (Schön et al. 2000). At present, the coexistence of forms with different breeding systems is reported in other ostracod species such as *Candonocypris novaezelandiae* (Chaplin 1992) and *Heterocypris barbara* (Rossi et al. 2007).

If transition is ancient, genetic differentiation may further increase, because of the accumulation of mutations generating new alleles that, without recombination, would be fixed in heterozygous genotype forms (Birky 1996). Accordingly, in unisexual populations of *E. virens*, genetic variability was related to the high number of alleles per locus and to high heterozygosity, generally coupled to significant heterozygtes excess and linkage disequilibrium. The large number of alleles we found, especially in unisexual populations from Northern Italy, supported the hypothesis that at least some lineages were reproducing parthenogeneti-

cally long enough to accumulate substantial variation through mutation. These results are in accordance with data obtained by sequencing in both nuclear ITS1 and mitochondrial COI loci: *E. virens* exhibits a very high substitution rate in both nuclear and mitochondrial DNA sequences (Schön et al. 2000). Moreover, this could explain the relatively high genetic differentiation we found among MLGs indicating that, in accordance with results from molecular data, the origin of some asexual lineage are much more ancient than others.

The identification of putative polyploid MLGs in all sexual populations of *E. virens* is a further suggestion of coexistence of lineages with different reproductive modes. Polypoidy and parthenogenesis were generally considered coupled phenomena (Suomalainen et al. 1987, Turgeon & Hebert 1994, 1995). Ploidity level cannot be directly evaluated from data and the number of polyploid genotypes in *E. virens* (14 %) is probably underestimated as only unbalanced heterozygosity could be identified. Nevertheless, this is considered a sufficient criterium to at least infer the presence of polyploidy (Little & Hebert 1994). If new clones arise through hybridization, at least polyploid parthenogens might be expected to be repeatedly generated in zones of sympatry between males and apomictic females (Chaplin 1992, Chaplin et al. 1994, Chaplin & Hebert 1997, Little 2005). The only unisexual population we found in Sicily was monomorphic and was made up of a polyploid MLG that could have originated, more or less recently, by hybridization or by internal genomic duplication (Little 2005). Transition to parthenogenesis and intraspecific hybridisation between parthenogenetic females and males of sexual relatives are involved in generating high genotypic diversity of *E. virens* (Turgeon & Hebert 1994, Hurst & Peck 1996, Schön et al. 2000, Rossi et al. 2007). Moreover, if the transition is due to spontaneous mutation that suppresses meiosis, this may give rise to lineages that produce a mix of sexual and parthenogenetic offspring or to asexual lineages that retain the occasional capability of producing functional males (Simon et al. 2003). These could "infect" offspring of sexual females and generate new parthenogenetic lineages. In *E. virens*, hybridisation could be underestimated either as a major route to parthenogenesis and as a source of genotypic diversity as reported in several parthenogenetic ostracod species (Tétart 1978, Havel et al. 1990b, Little & Hebert 1994, Chaplin et al. 1994, Turgeon & Hebert 1994, Turgeon & Hebert 1995). Tétart (1978) described at least seven different caryotypes in putative parthenogenetic French populations of *E. virens*. But, he reported that the majority of Cyprididae parthenogenetic species has maintained a diploid kariotype suggesting that parthenogenesis by polyploidy is a relatively recent acquired reproductive mode.

Only 26 % of different MLGs were detected in more than one population, generally located in the same geographical area. Relatively high level of variability among and within geographic regions may be the result of several processes: the stochastic differentiation of populations founded by a small number of individuals, differences in habitat specialisation and colonisation ability among genotypes, multiple colonisation by genotypes of different origin. The wide geographic distribution of MLGs and differences in genotypic diversity among populations described in *E. virens* present analogies with patterns observed in other ostracod species. In *Candonocypris novaezelandiae* from south-eastern Australia Chaplin & Ayre (1989, 1997) found a similar percentage of electrophoretic clones (23 %) in most of the sampled localities, 850 km apart. In *Cypridopsis vidua* populations from North America, Havel & Hebert (1989) observed that most clones are very rare and only 3 % of all clones detected are found from northern and eastern Canada to the southern United States. We described only four monoclonal populations: Bramhope (UK), Code d'Asino (Northern Italy), Morcuera (E) and Randazzo (Sicily). In the larger populations of Morcuera and Code d'Asino, lack of variation is confirmed in different seasons (Rossi, unpubl. data). These populations are isolated and located at high altitude, 1700 and 1275 m a.s.l, respectively. Their genetic uniformity may be the result of recent and/or occasional colonisation by a single lineage and/or the selective adaptation of a specific clone. As described in *Cypridopsis vidua* from Jamaica (Havel & Hebert 1989, Little & Hebert 1994) habitats that are geographically isolated or have been recently colonised should show low clonal diversity. The single MLG from Morcuera has been sampled in other Spanish populations and the single MLG from Code d'Asino is widespread in rice fields in Northern Italy. So, a local selective adaptation of these genotypes is not likely. The surprising close genetic relationship between the MLGs from Great Britain or Belgium and the MLGs from Sicily is in accordance with data on DNA sequences (Schön et al. 2000). This result may be explained by a recent colonisation of these areas by parthenogenetic lineages that have a common ancestor with sexual lineages from Sicily by, for instance, avian long distance dispersal of resting eggs (Bilton et al. 2001, Charalambidou & Santamaria 2002, Figuerola & Green 2002). In fact, parthenogenetic populations of *E. virens* can be started by a single resting

egg. Such desiccation-resistant propagules represent a very efficient passive dispersal mechanism (Baltanás 1998) and could explain the long-distance correlation between genetic and geographic distance. On the other hand, as a classical paradox in invertebrates inhabiting inland waters, high dispersal is coupled with reduced level of gene flow or, in parthenogenetic organisms, with genetic differentiation among nearby populations (Crease et al. 1990, Boileau & Hebert 1991, De Meester 1996, Vanoverbeke & De Meester 1998, De Meester et al. 2002). This phenomenon is explained by the stochastic differentiation of populations founded by small number of colonists that, through rapid adaptation and population growth, monopolise resources and hamper, through competition, the establishment of further colonists (De Meester et al. 2002). Meanwhile, clonal diversity could be maintained by the presence of specialised clonal lineages "frozen" along with adaptations for different niche partition of sexual population (frozen niche variation hypothesis) (Fox et al. 1996, Niklasson et al. 2004, Rossi et al. 2006). The parthenogenetic forms of *E. virens* are widespread at northern latitude as a consequences of historical factors (Pleistocene hypothesis) such as northern areas recolonisation after glaciations and higher colonisation ability of parthenogenetic lineages with respect to sexual ones (Baltanás 1998). Sexual lineages of *E. virens* have been found in the southern sampling areas, namely in Extremadura and in Sicily even in sympatry with parthenogenetic lineages. According to the destabilizing hybridization hypothesis, if gene flow between sexual and asexual lineages is maintained, this can result in the rapid displacement of one lineage by the other (Lynch 1984). In fact, sympatric sexuals and asexuals are not commonly observed in nature because, all else being equal, even on a short time scale, a sexual population will be outcompeted by an asexual population. The local displacement of sexually reproducing *Candonocypris novaezelandiae* by a conspecific parthenogen is described, for instance, in South-Eastern Australia (Chaplin 1993). Local displacement of sexually reproducing *E. virens* could be in progress but not yet over in Sicily, where sexual populations were described 25 years ago by Crosetti & Margaritora (1982). Alternatively, some ecological process must be at work: in temporary ponds that are kept in a permanently unstable state by frequent disturbance such as variable hydroperiod that, especially in Southern Europe, might promote the stable coexistence of amphimictic and apomictic lineages (Bell 1982, Booj & Guldemond 1984, Weinzierl et al. 1999, Hakoyama & Iwasa 2004). The same mechanism could prevent competitive exclusion and maintain clonal diversity in *E. virens* that, otherwise, was considered a species with reduced potential for niche differentiation (Weider 1992, Geiger et al. 1998).

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Data on MLGs and their frequency distribution in different populations are available upon request.

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