

# EVALUATION OF ELEVATED PLOIDY AND ASEXUAL REPRODUCTION AS ALTERNATIVE EXPLANATIONS FOR GEOGRAPHIC PARTHENOGENESIS IN *EUCYPRIS VIRENS* OSTRACODS

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Transitions from sexual to asexual reproduction are often coupled with elevations in ploidy. As a consequence, the importance of ploidy per se for the maintenance and spread of asexual populations is unclear. To examine the effects of ploidy and asexual reproduction as independent determinants of the success of asexual lineages, we sampled diploid sexual, diploid asexual, and triploid asexual *Eucypris virens* ostracods across a European wide range. Applying nuclear and mitochondrial markers, we found that *E. virens* consists of genetically highly differentiated diploid sexual populations, to the extent that these sexual clades could be considered as cryptic species. All sexual populations were found in southern Europe and North Africa and we found that both diploid asexual and triploid asexual lineages have originated multiple times from several sexual lineages. Therefore, the asexual lineages show a wide variety of genetic backgrounds and very strong population genetic structure across the wide geographic range. Finally, we found that triploid, but not diploid, asexual clones dominate habitats in northern Europe. The limited distribution of diploid asexual lineages, despite their shared ancestry with triploid asexual lineages, strongly suggests that the wider geographic distribution of triploids is due to elevated ploidy rather than to asexuality.

**KEY WORDS:** Colonization, geographic polyploidy, mitochondrial divergence, polyphyletic.

It is widely accepted that asexual organisms originate from sexual ancestors (Bell 1982). How and at what rate asexuality originates is important in predicting the capacity to become adapted to new environments, because high polyphyletic rates of origin could render parthenogenetic populations genetically as diverse as sexuals (Kondrashov 1993). A key confounding factor coupled to asexuality is uniparental reproduction that confers colonization advantages, that is, one single migrant individual is sufficient to found a new population. This has been postulated to lead to a wider geographic distribution of parthenogenetic lineages, which may not reflect adaptation to a wider range of environments (Vandel 1928). However, elevated ploidy is often associated with asexuality (Vandel 1928; Suomalainen et al. 1987) and may give ecological advantages in harsh environments, as is well documented for polyploid plants (Stebbins 1950; Levin 1983). Previous attempts to contrast the importance of asexual reproduction and elevated ploidy as explanations for geographic parthenogenesis have been complicated by an almost perfect match between the geographic distribution of reproductive modes and the geographic distribution of ploidy levels (Suomalainen et al. 1987; Jokela et al. 2003; Stenberg et al. 2003).

Transitions to higher ploidy often require special cytogenetic processes that circumvent the problems occurring during normal meiosis when uneven number of chromosomes attempt to pair. It is therefore not surprising that the great majority of uneven ploidy groups lack meiosis and reproduce asexually (Otto and Whitton 2000). In addition to merely having more DNA, advantages of polyploidy may result from frequent genome rearrangements (Soltis et al. 2004) and alteration of gene expression (Osborn et al. 2003). The greater ecological adaptability of polyploids may therefore enable them to invade and occupy new and harsh environments (Stebbins 1950). In line with this hypothesis, examples of ploidy elevation along a south-north cline are common (e.g., Ward et al. 1994; Little and Hebert 1997; Luttkhuizen et al. 2007). However, an unequivocal test of the direct advantage of polyploidy in harsh environments has proven difficult to produce because of other factors that are closely associated with polyploidy. For example, polyploids often originate through hybridization, which may lead to ecological differences between ancestral and derived lineages (Avisé et al. 1992). Hence, alternative explanations for the dominance of elevated ploidy in high latitudes include: (1) asexual polyploids simply colonize northern habitats more efficiently than sexual diploids, as discussed by Thompson and Whitton (2006) and Kearney (2005), or (2) polyploids are ecologically different from their ancestral lineages and are therefore found in the high-latitude habitats.

In this article, we survey diploid sexual, diploid asexual, and triploid asexual populations of *Eucypris virens* (Crustacea, Ostracoda) to contrast the advantages of ploidy and asexuality in ex-

plaining the geographic patterns. We characterize the origin, relatedness, and distribution of lineages using mitochondrial markers and the genetic diversity and population structure of each group using nuclear markers. By evaluating a system in which diploid and triploid asexual lineages and diploid sexual populations coexist, our goal is to separate the direct advantages of elevated ploidy and asexuality as explanations of invasion success and current geographical distribution.

## Materials and Methods

### STUDY SYSTEM

The freshwater ostracod *E. virens* is a winter species that lives in temporary ponds and survives dry-summer periods as desiccation-resistant eggs. The majority of dispersal is believed to take place as eggs. Sexual and asexual lineages are capable of producing both resting eggs and eggs that hatch immediately (Otero et al. 1998). *Eucypris virens* populations are highly polymorphic in both nuclear ITS1 and mitochondrial cytochrome oxidase subunit I, coding regions (COI) across Europe and there is clear evidence for multiple origins of asexual lineages (Schön et al. 2000). In agreement with sequence data, a previous study based on allozyme markers revealed remarkably high clonal diversities in local populations and occasional polyploidy (Rossi et al. 2008). The same study also confirmed apomictic parthenogenesis by breeding experiments. Asexual lineages are found across Europe whereas sexual reproduction is constrained to the Mediterranean region, including North Africa (Horne et al. 1998).

### SPECIMEN COLLECTION

Ostracod sampling of 21 arbitrary selected ponds was carried out in the hydrated periods of autumn–winter 2006 and winter–spring 2007 with the exception of site CCL (Table 1), which was sampled in spring 2005. All sites were given a three letter/number code that can be seen in Table 1. Two of these ponds, VLC and JAB, were sampled twice and three times, respectively, in the same season. In these repeated samples, the composition of multilocus allozyme genotypes did not change significantly between dates (the JAB sample consisted of one allozyme clone only) and seasonal data were therefore combined. Location of the sampling sites is shown in Figure 1. The animals were collected with a pipette or a hand net (250  $\mu\text{m}$ ) and samples were then either snap frozen and shipped on dry ice to the lab or sent alive.

### ALLOZYME ELECTROPHORESIS

We genotyped ostracods using cellulose acetate gel electrophoresis (CAGE), following the standard protocol of Hebert and Beaton (1993). The body of an individual ostracod was homogenized in 10  $\mu\text{l}$  of autoclaved nanopure water from which we took 7  $\mu\text{l}$  from fresh samples and 8  $\mu\text{l}$  from frozen samples for

**Table 1.** Names, abbreviations, and locations of the sampled ponds. "CC" refers to country code and "SITE" refers to codes used to refer to the population in the text. GPS coordinates ("LONG" longitude, "LAT" latitude) are given with degrees expressed as decimal fractions. "COI CLUSTER" contains information on mitochondrial haplotypes that were found in the sample. "COI CLUSTER" codes refer to clusters shown in Figure 3. Number of sequences is shown within brackets where superscript s, d, t, and ? represent, sexual, diploid asexual, triploid, and unresolved, respectively. \*Site is a connected pond complex and coordinates are presented for the most central pond.

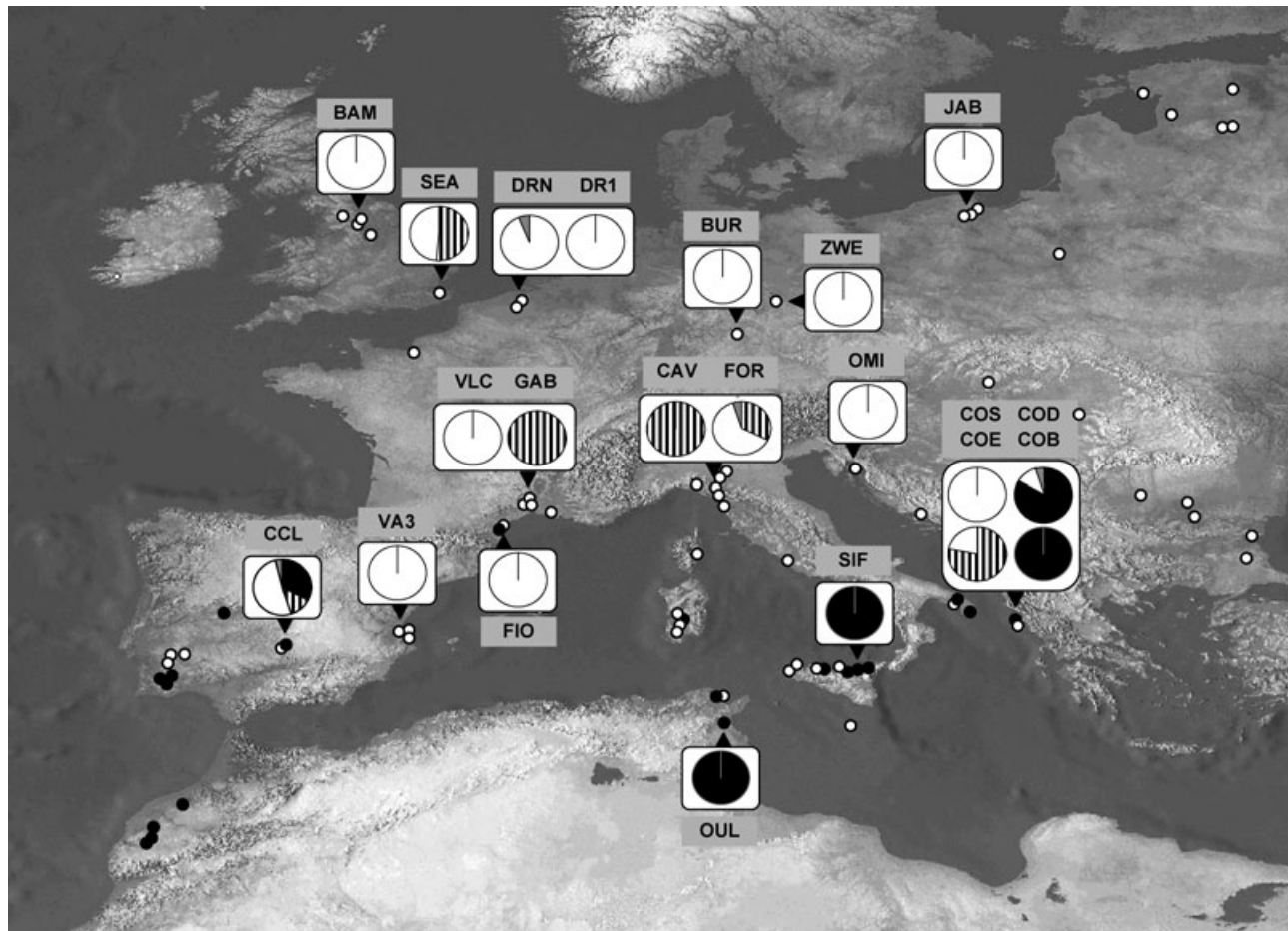
CC	SITE	COUNTRY	LONG.	LAT.	COI CLUSTER
TN	OUL	Tunisia	10.3183	36.0131	p30(11 <sup>s</sup> )
IT	SIF	Italy	14.6739	37.9358	p33(10 <sup>s</sup> )
ES	CCL*	Spain	-4.0644	38.8244	p8(3 <sup>d</sup> :4 <sup>t</sup> :1 <sup>?</sup> ); p23(19 <sup>s</sup> ); p25(3 <sup>d</sup> :8 <sup>t</sup> ); p31(19 <sup>t</sup> :1 <sup>?</sup> ); p32(2 <sup>t</sup> ); p33(2 <sup>t</sup> )
ES	VA3	Spain	-0.3083	39.3208	p31(2 <sup>t</sup> )
GR	COE	Greece	19.9100	39.4750	p29(2 <sup>d</sup> ); p31(1 <sup>t</sup> ); p35(1 <sup>d</sup> )
GR	COS	Greece	19.8508	39.6094	p28(2 <sup>t</sup> )
GR	COD	Greece	19.7986	39.6256	p25(1 <sup>t</sup> ); p29(1 <sup>t</sup> ); p31(1 <sup>t</sup> ); p33(1 <sup>t</sup> ); p34(6 <sup>s</sup> )
GR	COB	Greece	19.7856	39.6978	p34(21 <sup>s</sup> )
FR	FIO	France	2.9908	42.9242	p31(1 <sup>t</sup> )
FR	GAB	France	3.0689	43.0689	p12(1 <sup>d</sup> ); p25(1 <sup>d</sup> )
HR	OMI	Croatia	16.6958	43.4508	p33(2 <sup>t</sup> )
FR	VLC	France	3.8964	43.8089	p31(1 <sup>t</sup> )
IT	FOR	Italy	10.0928	44.0783	p28(3 <sup>d</sup> )
IT	CAV	Italy	10.0286	44.4183	p33(1 <sup>d</sup> )
DE	BUR	Germany	10.7283	49.8267	p11(1 <sup>t</sup> ); p28(2 <sup>t</sup> ); p29(1 <sup>t</sup> )
BE	DR1	Belgium	3.6653	51.0449	p31(1 <sup>t</sup> )
BE	DRN	Belgium	3.6766	51.0494	p17(1 <sup>t</sup> ); p28(1 <sup>t</sup> ); p29(1 <sup>?</sup> )
DE	ZWE	Germany	12.3286	51.1990	p29(3 <sup>t</sup> )
UK	SEA	UK	0.9883	51.3458	p31(2 <sup>d</sup> :8 <sup>t</sup> )
PL	JAB	Poland	20.7839	53.0208	p37(3 <sup>t</sup> )
UK	BAM	UK	-1.6214	53.9114	p25(3 <sup>t</sup> )

electrophoresis. The residue was again frozen at  $-80^{\circ}\text{C}$ . We found 11 polymorphic allozyme loci (all run with buffer system TG): AO (aldehyde oxidase EC 1.2.3.1), AD (aldehyde dehydrogenase EC 1.2.1.5), AAT (aspartate amino transferase EC 2.6.1.1), G6PDH (glucose-6-phosphate dehydrogenase EC 1.1.1.49), GPI (glucose-6-phosphate isomerase EC 5.3.1.9), IDH (isocitrate dehydrogenase EC 1.1.1.42), ME (malate dehydrogenase NADP<sup>+</sup> EC 1.1.1.40), MPI (mannose-6-phosphate isomerase EC 5.3.1.8), PEP (peptidase EC 3.4.11 or 3.4.13), PGM (phosphoglucomutase EC 5.4.2.2), and 6PGDH (6-phosphogluconate dehydrogenase EC 1.1.1.44). Due to the limited amount of material, especially for frozen specimens, only the five most variable loci AO, AAT, GPI, MPI, and PGM were used routinely. Of these five enzymes two, MPI and PGM, always appeared homozygous in males, whereas females from the same population had both homozygote and heterozygote genotypes, composed of the same alleles as found in males. Female genotypes were close to Hardy-Weinberg equilibrium. We therefore assumed that these loci are sex-linked and positioned on the X chromosome. Allele frequency calculations

were corrected thereafter and in the assignment of multilocus genotypes (MLGs), male genotypes were regarded as the same as a female homozygote genotype of the same allele. AO showed an unexplained excess of homozygotes in both males and females (>98%). This locus is therefore only included in MLG assignment and not in any diversity analysis (removal of AO only resulted in a reduction of 18 sexual and three triploid MLGs). None of the other loci used showed signs of gene duplications or other deviation from classic allozyme scoring (explained in Hebert and Beaton 1993). To improve scoring accuracy and resolve allelic identity we used a *Daphnia galeata* clone (clone G100 kept in culture by Piet Spaak, EAWAG; Switzerland) as a standard on each gel. In total, we genotyped 1602 individuals of which 1472 could be assigned to MLGs.

#### FLOW CYTOMETRY

To verify ploidy assignment from electrophoresis and to overcome the masking of ploidy level by homozygosity in allozyme analyses, we also estimated the relative DNA content with flow



**Figure 1.** Sampling locations covering the European distribution of *Eucypris virens*. Pie charts show frequency of sexuals (black), diploid asexuals (striped), and triploid asexuals (white) for each of the genotyped populations. Frequency calculations are based on individuals with definite ploidy and sex determination, the rest are unresolved (gray). Classification was done by allozyme genotyping, flow cytometry, and mitochondrial sequencing. Small dots in the map show the confirmed *E. virens* sites. Black dots indicate sexual sites and white dots asexual sites.

cytometry. DNA content was measured on a Partec Ploidy Analyzer PA-II equipped with an HBO mercury arc lamp that reads intensity of DAPI (4',6-diamidino-2-phenylindole) stained DNA per particle. The 2  $\mu$ l (frozen samples) or 3  $\mu$ l (fresh samples) residues from allozyme analysis were incubated for 55 sec in 100  $\mu$ l of CyStain UV Precise T Extraction Buffer (Partec, Muenster, Germany), then 900  $\mu$ l of CyStain UV Precise T Staining Buffer (Partec) was added into the tube before running the solution through a Partec CellTrics 50  $\mu$ m mesh (CyStain UV Precise T Extraction Buffer and CyStain UV Precise T Staining Buffer were sometimes replaced with the ready-to-use mixture CyStain UV Ploidy (Partec)). The sample was either directly analyzed on the machine or kept in the dark for a maximum of 20 min before analysis (this did not effect the DNA content reading). As a size standard, we used the same *D. galeata* clone as for allozyme control. The *Daphnia* sample used as a size reference was prepared in the same way as the test samples. The reference sample was run through the instrument at the beginning of each series of measure-

ments. Alternatively, a suspension of *Daphnia* nuclei was added to the test samples before running through the machine.

Relative DNA content was calculated as the ratio of mean peak value and the corresponding reference value. All distinct peaks were included as there was a clear gap between diploid and triploid DNA contents (mean CV = 6.96 and mean particles counted = 2240). Two hundred and thirty-seven genotyped individuals from 95 MLGs (20 homozygote MLGs) were analyzed. These measurements mostly verified the allozyme-estimated triploid/diploid classifications, as the error rate of ploidy assignment based on allozymes was 2.9% for the MLG tested. The majority of errors in ploidy assignment occurred when a true triploid MLG was scored as a diploid with allozymes. Relative DNA content of both diploids and triploids was found to be variable (S. Adolfsson et al. unpubl. data), but individuals of the same MLG always belonged to the same ploidy class and therefore we assume that an individual's ploidy is known if one or several individuals of the same MLG have been analyzed for DNA content.

This prediction was confirmed by repeated flow cytometry measurements of individuals with the same MLG (performed on 143 individuals from 25 MLGs: median: 2, range: 2–25, individuals per MLG). However, we did not extrapolate between populations. Overall, we resolved ploidy for 57% of the homozygote MLGs with flow cytometry (diploids 81% vs. triploids 19%). The majority of the remaining homozygote MLGs were classified as sexuals based on the analyses of allele frequencies, and were assumed to be diploid (only two asexual MLGs remained unresolved).

### COI SEQUENCING

We used the allozyme extraction residue, when not used for flow cytometry, for COI sequencing. We extracted DNA applying 25  $\mu$ l/17  $\mu$ l (depending on the amount of homogenate) of extraction buffer (10 mM Tris\_HCl [pH 8.3], 50 mM KCl, 0.005% Tween20, 0.005% Nonidet40 (Sigma Aldrich, St. Louis, MO)). Samples were incubated for 1 h before adding 1  $\mu$ l Proteinase K, followed by incubation over night at 50°C, and a termination step at 95°C for 10 min. Two primer pairs were used for PCR amplification: HCO (forward) 5'-TAAACTTCAGGGTGACCAAAAATCA-3' with LCO (reverse) 5'-GGTCAACAATCATAAAGATATTGG-3' (658 bp) (Folmer et al. 1994); and FMCO 5'-TAGGACAGCCRGATCWCT-3' with RMCO 5'-CGGTCTGTTAAWAGCATWGTGA-3'. The latter pair was designed for a few populations, which could not be amplified with the universal primer pair, using conserved regions in sequences already obtained. PCR amplifications using primer pair HCO/LCO were run as: 3 min of denaturation at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 54.5°C, 1.5 min at 72°C, and a final extension step of 10 min at 72°C. The 20  $\mu$ l volume reactions contained 0.5 U recombinant *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 1  $\times$  PCR Buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 2 mM dNTP, 10  $\mu$ M of each primer, 1.5 mM MgCl<sub>2</sub>, and ~8 ng template DNA, although DNA yield was variable. PCR amplifications using primers FMCO/RMCO were conducted as described for the universal primers, except that a total of 37 cycles was used with an annealing temperature of 48°C or 45°C. This was done using the HotStar Taq Master Mix Plus Kit (Qiagen, Hilden, Germany) applying the manufacturer's protocol. Amplification products were run on agarose gels and stained with SYBR Safe (Invitrogen). Thereafter, PCR cleanup was done applying the ExoSAP-IT (GE Healthcare, Waukesha, WI) system or for a few samples by following the protocol of the QIAquick PCR Purification Kit (Qiagen) or the GFX<sup>TM</sup> PCR DNA & Gel Band purification kit (GE Healthcare). The cleaned amplification products were prepared for sequencing using the ABI cycle sequencing kit manual and sequenced in both directions using the ABI BigDye Terminator version 1.1. kit (Applied Biosystems, Foster City, CA) on an ABI 3730 $\times$ 1/ABI 3130 $\times$ 1 Genetic Analyzer with the PCR primers.

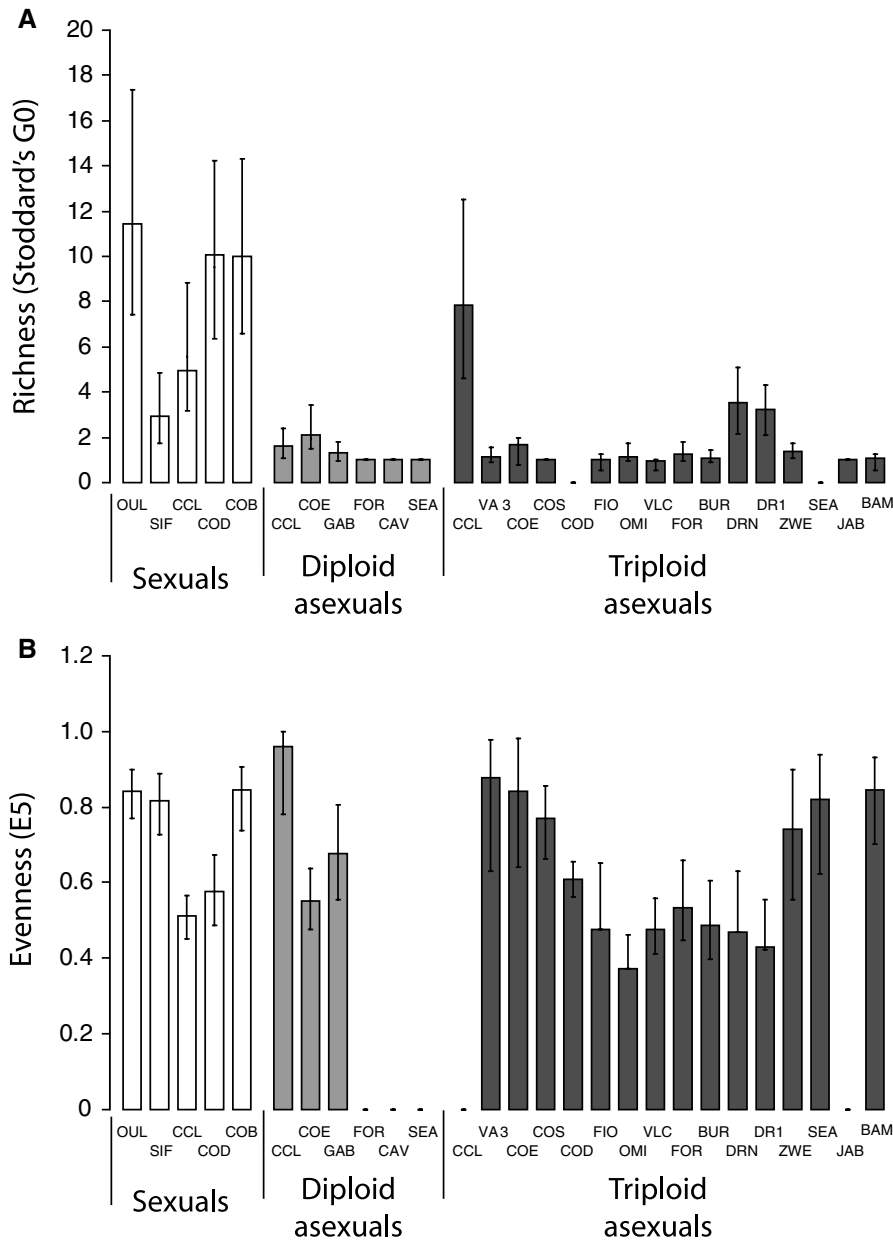
### ANALYSIS

Males were separated from females on the basis of morphology. The male copulatory apparatus is readily visible using a low-power microscope. However, so far, no obvious morphological differences have been recorded between sexual and asexual, or between diploid and triploid *E. virens* females. Triploid individuals can easily be distinguished from diploids, both from allozyme band intensities and by DNA content. It is more difficult to separate sexual females from diploid asexual females. We assigned asexuality based on the frequency distribution of female MLGs, assuming Hardy–Weinberg equilibrium and with guidance from alleles present in males (where they occur). Assignments were subsequently checked with the program genclone version 2.0 (Arnaud-Haond and Belkhir 2007) in which MLGs with a  $P_{\text{sex}}$  value  $\geq 0.05$  were considered sexual,  $P_{\text{sex}}$  values  $< 0.05$  and  $\geq 0.01$  were left unresolved, and  $P_{\text{sex}}$  value  $< 0.01$  distinguished diploid asexual MLGs. Only five MLGs were classified differently using the two methods, in these cases the  $P_{\text{sex}}$  value was used.

For each pond, we calculated the proportions of sexual, asexual diploid, and asexual triploid individuals (Fig. 1). We used Spearman rank correlations of frequencies with latitude to test two predictions: (1) asexuals (diploid or triploid) are more common in the north than sexuals, (2) triploids are more common in the north than diploids (sexual or asexuals).

Genotypic diversity of the target groups (i.e., diploid sexuals, diploid asexuals, and triploids) was measured using the Stoddart-index  $G_0$  (Stoddart and Taylor 1988).  $G_0$  is a measure of genotypic richness ranging from 1 (single genotype) to  $N$  (where  $N$  is the sample size, i.e., each individual belongs to a different MLG). The upper limit of  $G_0$  is a function of sample size when  $G_0 \sim N$ . This is problematic if one needs to compare samples of different size (Grunwald et al. 2003). To circumvent this caveat, we randomly selected, with replacement, a subsample of 20 individuals from each observed sample. After 1000 bootstraps, we recorded the median  $G_0$  and the 2.5% and 97.5% percentiles. Five populations had a sample size smaller than 20. For these we performed 1000 bootstraps using the full sample to calculate the median and percentiles. Three of these populations were monoclonal, or with low genotypic diversity (population FOR diploid asexual, SEA diploid asexual, and COE triploid), and could be compared to subsampled populations (see Fig. 2). For COD triploids and SEA triploids, which had a low sample size and relatively high genotypic diversity, we report the bootstrapped medians and percentiles but make no direct comparisons to other populations.

We also calculated genotypic evenness. Evenness describes how genotypes are distributed within a sample. Low values (min = 0) describe populations with one or few clones of variable frequency and high values (max = 1) describe populations in which genotypes are equally common. We calculated evenness using the



**Figure 2.** Median genotypic richness (Stoddard's  $G_0$ ) and genotypic evenness ( $E_5$ ) of genotyped populations. Sexual populations are shown in white, diploid asexuals in light gray, and triploids in dark gray. The populations are arranged by latitude within each of the groups from south to north. Medians were estimated based on 1000 bootstrap samples of the multilocus genotypes. Error bars show the 2.5% and 97.5% percentiles of the bootstrap distribution.

index  $E_5$  described in Ludwig and Reynolds (1988).  $E_5$  is less affected by sample size differences than other measures (Grunwald et al. 2003). Median and percentiles were obtained by 1000 randomizations, with replacement (Fig. 2). We used Matlab 7.0.4 (MathWorks, Natick, MA) for randomizations and calculations of both richness and evenness indices. The Mann–Whitney non-parametric test was later applied to determine the statistical significance of differences in genotype richness or evenness between groups. COD triploids and SEA triploids were not included in the genotypic richness differentiation test.

Diploid gene diversities within target groups by site were calculated in GENEPOP version 3.3 (Raymond and Rousset 1995), whereas triploid gene diversities were calculated manually using the formula  $1 - \sum P_i^2$ , where  $P_i$  is the frequency of the  $i$ th allele in the population (Table S1). A Kruskal–Wallis H test was performed to detect differences in mean gene diversity between reproductive mode/ploidy groups.

Genetic structure of the populations was estimated using  $F_{ST}$ . We used GENEPOP version 3.3 to estimate  $F_{ST}$  and test the significance of population differentiation between diploid populations

(Raymond and Rousset 1995). Population genetic structure of triploid populations was estimated using R code that was kindly provided by J. Goudet (University of Lausanne, Switzerland). This R code partitions variance in allele frequencies among populations for triploids. The accompanying tests of significance were performed by randomly permuting individuals among populations.

We calculated genetic similarity among MLGs using the Tomiuk and Loeschcke Identity measurement (Tomiuk and Loeschcke 1991, 1995, 1996). This identity measure is one of the few methods that can be used to calculate genetic similarity values between sexual, asexual, and different ploidy genotypes. We included all MLGs that were observed more than once and defined each MLG as a unit so that one value of similarity was calculated between every pair of MLGs. In contrast to the previously described  $F_{ST}$  analysis, this measurement defines genetic similarity directly among genotypes, instead of between individuals, and does not take into account genotype frequencies in the population. We used this approach to compare identity between MLGs of target groups (sexual, diploid asexuals, and triploids) among- and within-sites (Table 2). In this context, we were interested in whether asexual clones from one pond are more similar to one another than they are to clones that come from other ponds. Asexual genetic similarity was also compared to sexual MLG similarity among- and within-ponds to test whether patterns of dispersal and/or colonization differ between reproductive modes. Genotypic identity measures were calculated with POPDIST (version 1.1.1) (Guldbrandtsen et al. 2000) choosing the Tomiuk and Loeschcke Identity. Means were calculated as mean of all pairwise identities within-ponds and as mean of all pairwise identities among-ponds. To determine significance, the observed mean identity differences (mean among-site subtracted from mean within-

site), were compared to 1000 mean identity differences obtained by permuting genotypes across sites and calculating within- and among-site mean identities for each permutation. These permutations were performed for each target group separately, that is, sexual, diploid asexual, and triploid. In addition, we performed 1000 genotype permutations among-sites including all target groups, after which expected identity means within-site and among-site comparisons were calculated (Table 2). Permutations were done in Matlab 7.0.4.

We included our COI sequences into a larger dataset of *E. virens* sequences to assess the phylogenetic pattern of ploidy and asexuality. The phylogenetic methods and the detailed analysis of the complete dataset are described in Bode et al. (2009). The study by Bode et al. describes the phylogeny of the group of sexual and asexual ostracods that are morphologically classified as *E. virens* and extends the data presented here by including more sampling sites and an additional mitochondrial marker (16S). A Bayesian method was used for phylogenetic analysis of the mtDNA sequence data and carried out in BEAST version 1.4.8 (Drummond and Rambaut 2007). The implemented substitution model chosen was HKY (Hasegawa–Kishino–Yano) with six Gamma categories and three partitioned codon positions under the assumption of a strict molecular clock. The likelihood of this model exceeded two other implemented strict clock models: general time reversible with gamma distribution and invariable sites (GTR +  $\Gamma$  + I) and SRD06 (HKY with the first and second position partitioned per codon). Default settings of *Beauti* version 1.4.8. parameters were used except for Jeffrey's prior distribution which was set to 5.0. We applied a Yule algorithm describing a speciation process rather than a population-based coalescent process (as preliminary trees showed deep branches). The final phylogeny was based on three combined independent runs (1,000,000 generations each, within which we sampled every 10,000th tree and excluded the first 1000 trees as burnin) including the outgroup taxa *Tonnacypris lutaria* and *Eucypris pigra* (Fig. 3). Later, the algorithm of Pons et al. (2006) was applied to the phylogeny to objectively delimit species-like boundaries, here referred to as clusters. This method determines the most likely transition from the speciation process to a coalescent process.

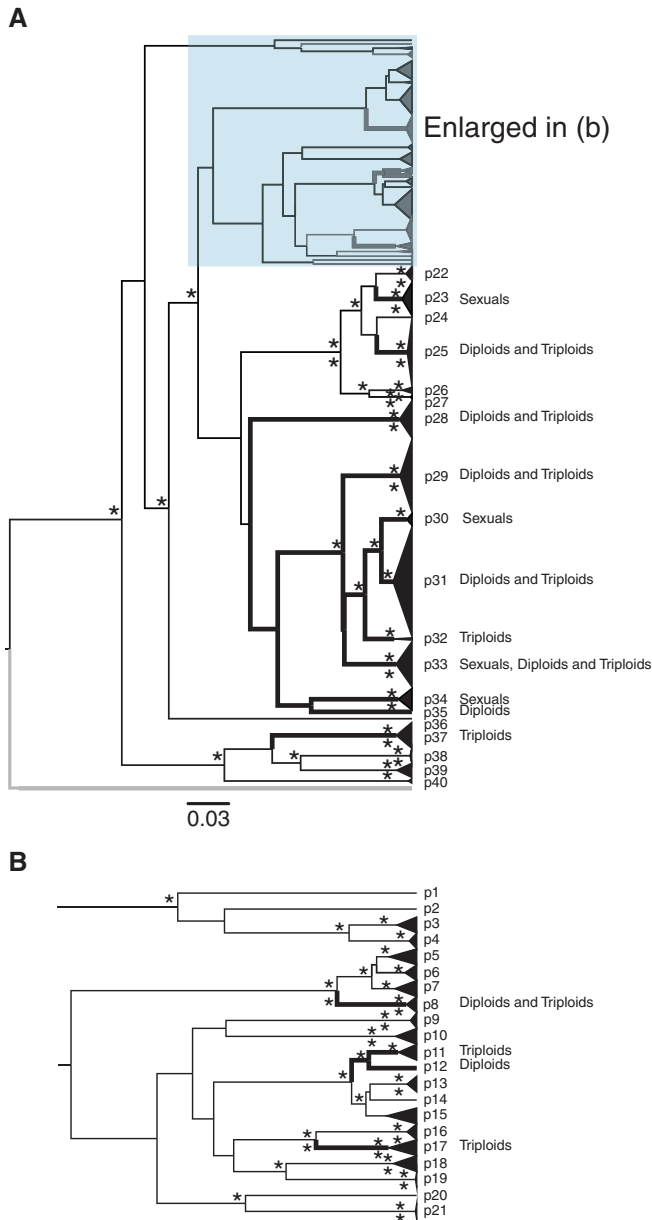
**Table 2.** Tomiuk and Loeschcke Identities ("T&L IDENTITY", mean:  $\bar{x}$ , one standard deviation: SD) among MLGs within- (W) and among- (A) sites are presented in the left three columns (a value of 1 describes full identity). Observed sexual (s), diploid asexual (d), and triploid (t) identities were compared to 1000 means obtained by genotype permutations among-sites (all s, d and t genotypes included). "NS" indicates  $P$ -values that were not significant at  $P < 0.05$  (indicates lower than simulated mean) or  $P > 0.95$  (indicates higher than simulated mean).

	T&L IDENTITY		
	$\bar{x}$	SD	$P$
$W_s$	0.78	0.13	1
$W_d$	0.55	0.22	NS
$W_t$	0.63	0.19	1
$A_s$	0.44	0.20	0.002
$A_d$	0.39	0.21	0.03
$A_t$	0.55	0.20	1

## Results

### GEOGRAPHIC PATTERN OF PLOIDY AND MODE OF REPRODUCTION

The results of our survey confirmed those of previous studies reporting a strong geographic pattern in the distribution of asexual lineages and sexual populations. Sexual reproduction was completely restricted to the Mediterranean area. Across all sampled sites, the frequency of sexual individuals was negatively correlated with latitude (Spearman  $r = -0.625$ ,  $n = 21$ ,  $P = 0.002$ ). Diploid



**Figure 3.** (A) Bayesian phylogenetic strict clock tree based on *Eucypris virens* mitochondrial COI sequences. (B) Extension of clusters p1–p21. Triangles at the tips of branches represent clusters determined by sequence based species delimitation (see methods). Lines without triangles are unique sequences that form one cluster unit. Posterior probabilities (above nodes) and bootstrap values (below nodes) higher than 0.95 are indicated by a star. The bootstrap values were obtained by *PhyML* version 2.4.4 with 500 bootstrap replicates. Bold lines indicate clusters in which we have both COI sequence and allozyme/flow cytometry information. Label next to the cluster number specifies if sequences in that cluster contain diploid sexual individuals (“Sexuals”), diploid asexual individuals (“Diploids”), or triploid asexual individuals (“Triploids”). Outgroup is shown with a gray bold line.

asexual genotypes, with the exception of one homozygous diploid clone found in Kent, UK, were found south of the Alps around the northern rim of the Mediterranean. This leaves the northern part of Europe dominated by triploid clones (see Fig. 1). However, triploid abundance in the north does not imply that they are restricted to higher latitudes; on the contrary triploids were often found in the same area as sexual and diploid asexual populations. The correlation between frequency of triploids and latitude was significantly positive (Spearman  $r = 0.472$ ,  $n = 21$ ,  $P = 0.031$ ).

Sixteen sites of 21 had only one reproductive mode/ploidy, but we also observed five mixed sites where sexual, diploid asexual, and triploid asexual individuals coexisted (Fig. 1). We did not find any MLGs that had a wide geographic range.

### ALLOZYME DIVERSITY

Independent of ploidy, the genotypic diversity of asexual *E. virens* populations was lower than that of the sexual populations, with five completely monoclonal populations (CAV diploid asex, COS triploid, FOR diploid asex, JAB triploid, and SEA diploid asex) and several with very low numbers of genotypes (Fig. 2) (sexual vs. diploid asexual: Mann–Whitney  $U < 0.001$ ,  $P = 0.006$ ; sexual vs. triploid: Mann–Whitney  $U = 4$ ,  $P = 0.004$ , and diploid asexual vs. triploid: Mann–Whitney  $U = 37$ ,  $P = 0.678$ ). Two populations, COD and SEA, had few triploid individuals but relatively many triploid genotypes. For these populations, the median  $G_0$  reached 2.37 (2.5 and 97.5 percentiles: 1.16 and 3.56, respectively) and 4.00 (2.5 and 97.5 percentiles: 2.29 and 5.82, respectively) based on sample size of eight and 16 individuals, respectively. However, due to the small sample size these values may be misleading when compared to other populations (Fig. 2). Clonal evenness was always high and only diploid asexuals had a lower mean evenness than sexuals (sexual vs. diploid asexual: Mann–Whitney  $U = 3$ ,  $P = 0.027$ ; sexual vs. triploid: Mann–Whitney  $U = 19$ ,  $P = 0.083$ , and diploid asexual vs. triploid: Mann–Whitney  $U = 32$ ,  $P = 0.235$ ).

Although asexual genotypic diversity was low, this was not the case for asexual gene diversities. Both asexual groups showed similar gene diversities to sexuals (Kruskal–Wallis:  $\chi^2 = 0.673$ ,  $P = 0.714$ ) (Table S1). Only a few multilocus genotypes were shared among populations: 8% of all MLGs were shared among sexual sites, 0.4% between sexual and diploid asexual sites, 0% between diploid asexual sites, and 0.4% between triploid sites. However, we detected no alleles that were restricted to a specific geographic location (Table S1). Pairwise  $F_{ST}$  estimates were very high. Estimates for sexual populations were higher than 0.26 (0.26–0.60; mean 0.43), except for one pair of Greek populations with  $F_{ST} = 0.03$ . Similarly, estimates among diploid asexual populations ranged between 0.26 and 0.78 (mean 0.52) and among

triploid populations ranged between 0.09 and 0.79 (mean 0.42). All pairwise  $F_{ST}$  were significantly larger than 0.

We hypothesized that if no structure was seen in genetic similarity among MLGs within- versus among-populations, this would indicate admixture and high dispersal rates. The Tomiuk and Loeschcke mean identities among genotypes among-sites were significantly lower than identities within-sites (mean within-site–mean among-site  $> 0$ ) in all comparisons (observed difference vs. difference under randomization: sexual:  $P = 0.001$ ; diploid asexual:  $P = 0.034$ ; triploid:  $P = 0.003$ ). Furthermore, the mean identity of triploid genotypes among-sites was significantly higher than the mean identity of diploids among-sites (Table 2). Within-sites, the observed mean identities for both triploid and sexual MLGs were significantly higher than expected under complete genotype randomization among-sites (all target groups included). Taking these observations together, the difference in mean identity within- versus among-sites was significantly lower in triploids than in sexuals.

#### ORIGIN AND DIVERGENCE OF REPRODUCTIVE MODE/PLOIDY GROUPS

Figure 3 illustrates the relationships among 374 COI sequences from *E. virens* spread across Europe (Bode et al., 2009) (GenBank accession no. GQ914281–GQ914731 and GU186126–GU186152. For cross-referencing to sex and ploidy see Table S2.) One hundred and fifty-nine sequences were linked to allozyme genotypes and estimations of ploidy. COI cluster representation per pond can be seen in Table 1 and COI clusters containing any or several individuals from listed ponds are marked with bold branches in the phylogeny. Populations analyzed in this article fall into 15 clusters (three purely sexual, one mixed, and 11 purely asexual clusters).

We observed that mitochondrial lineages in sexual individuals were always local whereas the closely related asexual lineages can be widespread across Europe (Fig. 3 and Table 1. See also Bode et al., 2009). In sites where sexual and asexual females coexist, the individuals classified as sexual with allozymes and flow cytometry always clustered with males of the same site in the mtDNA tree whereas females classified as asexual clustered elsewhere. Thus, allozyme data strengthen the inference of sexual versus asexual status of females based on the presence of males within clusters. The sexual samples with allozyme data form four distinct clusters (Fig. 3). MtDNA sequence divergence among these sexual clusters ranges from 10% to 13% and is clearly in the range of divergence levels observed between cryptic species (a 3%  $P$ -distance has been suggested as a threshold for species status by Hebert et al. (2003)).

These results suggest that asexuality has arisen several times (Fig. 3) but is this true for both asexual ploidy groups? When we examined the clusters containing sequences in which we know the

ploidy, we were able to infer several origins of asexual diploid and triploid lineages (see Fig. 3). Of the 12 clusters with allozyme-linked asexual representatives, six have representatives of both ploidy levels. The remaining six clusters (four triploid only and two diploid asexual only) have low numbers of allozyme-linked sequences ( $\leq 3$ ), therefore we could easily have missed the absent group. Several asexual clusters include individuals from multiple sites (Table 1): one contains diploid asexual genotypes and five contain triploid genotypes from several locations. Likewise, many of the sites are populated by asexual lineages from more than one cluster (Table 1): three sites with diploid asexual and four sites with triploid asexual genotypes. These observations can be explained at the MLG level. That is, asexual MLGs always have very close mitochondrial sequence similarity, which means they are found in the same cluster, but different MLGs within-sites can be greatly diverged in mtDNA sequence.

### Discussion

We found several interesting, previously not well-understood patterns of origin and geographic distribution of ploidy in this group of freshwater ostracods. We will first discuss the origin of asexual lineages and higher ploidy. In short, we found the origin of asexual reproduction to be polyphyletic and involve both diploid and triploid asexual lineages within the same clades. We will then discuss the relatedness and geography of sexual, diploid asexual, and triploid asexual reproductive mode distributions showing that, in these phylogenetically replicated groups, the triploid asexual lineages seem to be the group that has spread to northern habitats suggesting that elevated ploidy, not asexuality, has direct advantages in new environments.

#### MULTIPLE COMMON ORIGINS OF ASEXUAL PLOIDY GROUPS

We aimed to describe the geographic distribution of polyploidy, in the context of the origin and colonization history of individual lineages, to gain insight into the generality of the observed geographic pattern and the processes that may have generated it. We have shown that extant diploid and triploid asexual lineages of *E. virens* have originated through repeated transitions and are polyphyletic (Fig. 3). Multiple origins of polyploidy have previously been reported in many systems, including ostracods (Chaplin and Hebert 1997), snails (Johnson and Bragg 1999), weevils (Normark 1996), and several plant species (Soltis and Soltis 1993). Of the 12 asexual clusters that we identified using mtDNA sequence data, 10 are apparently derived from a separate origin of parthenogenesis, whereas two clusters (p11 and p12) appear to be the result of subsequent diversification. Only two of these asexual clusters do not have a triploid representative, indicating at least 10 independent transitions to polyploidy. As all sampled clusters containing

more than three sequences have both diploid and triploid asexuals, we conclude that the transitions to asexual reproduction do not have to involve an increase in ploidy although they commonly do, and that the transitions have occurred throughout this taxon, within each of several highly diverged sexual clades (Fig. 3). The consistency in this kind of transition involving asexuals in ancestral and elevated ploidy levels has never been described before in animals. The mitochondrial divergence between sexual ancestor populations in combination with concurrent origins has inevitably led to asexual lineages with similarly high levels of divergence.

If triploid lineages commonly share sexual ancestry with diploid asexual lineages, one has to question the evolutionary isolation, that is, origin and independence, of the asexual lineages. We found no difference in allozyme allele frequency similarities between triploids and their most closely related sexuals compared to similarities between triploids and their most closely related diploid asexual genotypes (data not shown). Therefore, two pathways to triploidy remain plausible, they could originate either (1) through fertilization of an unreduced sexual gamete or (2) through fertilization of an unreduced diploid asexual gamete. The first pathway seems to dominate in *Potamopyrgus* snails (Wallace 1992) and *Trichoniscus* isopods (Christensen 1983) in which diploid asexuals are not reported. Hybridization, the second pathway, between diploid asexual and sexual gametes is widely supported in ostracods, for example, *Heterocypris incongruens* and *Cypricercus reticulatus* (Turgeon and Hebert 1994, 1995; Johnson et al. 1999).

#### CURRENT GEOGRAPHIC RANGE AND PATTERN OF COLONIZATION

To separate the effects of ploidy from mode of reproduction as an explanation of wide geographic range, we first examined the association between ploidy and geographic parthenogenesis. Then, we specifically asked if triploid asexual genotypes have a different geographic distribution from diploid asexual genotypes, which would reveal whether ploidy or asexuality is more important for the successful colonization of a wide geographic range. We found that triploids were common all over Europe whereas diploids were almost exclusively confined to the area around the Mediterranean, supporting the original theory predicting a wide geographic spread of triploids (Fig. 1). Significant correlations were found between latitude and frequency of triploidy (positive) and between latitude and sexual reproduction (negative). The limited distribution of diploid asexuals despite common ancestry with triploid asexuals suggests their relatively low colonization success. However, the generation of diploid asexual lineages may not be as frequent as that of triploid lineages, especially if hybridization between diploid asexuals and sexuals is common.

As we found no evidence of widespread genotypes, we conclude that the geographic pattern was not caused by success-

ful general-purpose genotypes that would be competing globally (Lynch 1984; Parker and Niklasson 1995). Lack of globally successful clones does not mean that clonal selection cannot operate at the local level. We observed low genotypic diversity in many triploid and, especially, diploid asexual populations. In fact, only a few triploid populations harbored genotypic richness comparable to sexuals (Fig. 2). Such a pattern could be due to colonization barriers reducing the likelihood that the same pond is colonized by multiple clones, or by strong interclonal competition reducing genotypic diversity locally. Our results do not support the existence of colonization barriers, as we have evidence for multiple colonizations of both northern and southern sites. That is, we repeatedly observed very distantly related clones coexisting locally and the co-occurrence of several mtDNA clusters within populations (Table 1). High colonization rates are also supported by the analysis comparing MLG mean identity between- and within-sites. For triploids, we found only a small difference in average identity within- and between-sites, suggesting that the colonization rates are high (Table 2). Furthermore, experimental work on interclonal competition supports the idea that competition can rapidly alter clonal structure, strongly reducing clonal diversity (Martins et al., in press).

We assessed the genetic diversity and structure of asexual lineages in local populations to search for correlations between diversity and latitude (Table S1). According to our results, the allele patterns were poorly explained by geographic location,  $F_{ST}$  values were high between geographically close populations, and the overall high gene diversity did not depend on the reproductive mode or a cline in latitude. This is in agreement with a previous *E. virens* clonal structure study by Rossi et al. (2008), but in contrast to this earlier study we found a well-supported latitudinal cline in ploidy possibly due to our broader sampling range, in particular having more northern sites. We have also extended previous studies by reporting triploid genetic diversity.

In conclusion, our results show that *E. virens* experienced a relatively large number of independent shifts in its reproductive mode and ploidy level. The triploid lineages appear to have been better in colonizing the northern parts of Europe, generating the observed pattern of geographic parthenogenesis. This suggests that ploidy elevation promotes range expansion of these lineages more effectively than parthenogenesis per se.

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## Supporting Information

The following supporting information is available for this article:

**Table S1.** Identified alleles and gene diversities of all populations.

**Table S2.** The information in this table is linked to the sequence names in Genbank, which are coded as follows: COUNTRY CODE\_ISOLATE CODE DNA NUMBER.

Supporting Information may be found in the online version of this article.

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1 **Supporting information**

2 Table S.1. Identified alleles and gene diversities of all populations. The first five populations are  
 3 sexual, followed by six diploid asexual populations and the last 16 populations are triploid  
 4 asexual populations. Populations are organised geographically from south to north within each  
 5 group. N = number of genotyped individuals.

6

7

		ALLELES				GENE DIV				
POP	N	GPI	AAT	MPI	PGM	GPI	AAT	MPI	PGM	$\bar{x}$
OUL	58	2:3:4	1:2	1:2:3:4	3:4:5	0.54	0.30	0.56	0.49	0.47
SIF	66	1:3	2	4:5	3:4	0.25	0.00	0.29	0.18	0.18
CCL	222	2:3:4	1:2:3	2:3:4	2:3:4	0.05	0.16	0.54	0.24	0.25
COD	55	1:2:3:4	2:3	2:3:4	3:4:5	0.47	0.04	0.45	0.50	0.37
COB	66	1:2:4	2	1:2:3	4:5	0.60	0.00	0.44	0.50	0.39
CCL	84	3:4:5	1:2	1:2:3	2:3	0.68	0.37	0.14	0.08	0.32
COE	25	3:4:5	1:2	2:4:5	3:4:5	0.58	0.50	0.63	0.68	0.59
GAB	66	3:4:5	2:3	1:2:3	2:3	0.63	0.28	0.30	0.15	0.34
FOR	13	2:3	2	3:4	2:4	0.50	0.00	0.50	0.50	0.38
CAV	32	1:3	2	4	3:4	0.50	0.00	0.00	0.50	0.25
SEA	17	2	3	4	3	0.00	0.00	0.00	0.00	0.00
CCL	349	1:2:3:4:5:6	1:2:3	1:2:3:4:5	2:3:4:5	0.60	0.54	0.15	0.64	0.48

22

23

24	VA3	44	1:2:3:4	2:3	2:3:4	3:4:5	0.67	0.13	0.46	0.67	0.48
25	COE	7	1:3:4:5	1:2:3	1:3:4	3:4	0.60	0.44	0.44	0.49	0.49
26	COS	32	2:4	3	3	3:4:5	0.44	0.00	0.00	0.67	0.28
27	COD	8	2:3:4:5	1:2	2:3:4:5	3:4	0.57	0.22	0.62	0.38	0.44
28	FIO	20	2:3	3	3:4	3:4	0.06	0.00	0.10	0.03	0.05
29	OMI	60	3:4:5	2	2:3:4	3:4:5	0.44	0.00	0.49	0.05	0.25
30	VLC	71	1:3:4	3	3:4	3:4:5	0.46	0.00	0.02	0.46	0.23
31	FOR	25	3:5	1:2	2:3:4	3:4	0.44	0.05	0.48	0.44	0.35
32	BUR	33	1:2:3	2:3	1:2:3:4	2:3:4	0.45	0.06	0.55	0.51	0.39
33	DR1	30	2:3:4	1:2:3	2:3:4:5	2:3:4	0.24	0.52	0.72	0.62	0.52
34	DRN	61	2:3:5	1:2:3	1:2:3:4	2:3:4	0.20	0.53	0.65	0.52	0.48
35	ZWE	33	1:2:3:4:5	2	3:4	2:3:4	0.74	0.00	0.13	0.58	0.36
36	SEA	16	1:2	2:3	3:4	3:4:5	0.22	0.38	0.50	0.46	0.39
37	JAB	33	2:3	2	1:2	1:2:5	0.44	0.00	0.44	0.67	0.39
38	BAM	22	2:3	2:3	1:2:3:4	2:3:4	0.09	0.09	0.48	0.45	0.28

39  
40 Table S.2. The information in this table is linked to the sequence names in Genbank, which are  
41 coded as follows: COUNTRY CODE\_ISOLATE CODE DNA NUMBER. Isolate codes CC3-5  
42 and CL1-2 corresponds to site CCL. The additional columns contain information on reproductive  
43 mode and ploidy level.

44

COUNTRY	ISOLATE	DNA NR	SEX	PLOIDY
ES	CC3	302	asex	?
ES	CC3	303	asex	Triploid
ES	CC3	304	asex	Diploid
ES	CC3	305	sex	Diploid
ES	CC3	306	asex	Triploid
ES	CC3	307	sex	Diploid

ES	CC3	309	asex	Triploid
ES	CC3	310	asex	Triploid
ES	CC3	312	asex	Diploid
ES	CC3	313	sex	Diploid
ES	CC3	314	asex	Triploid
ES	CC3	316	sex	Diploid
ES	CC3	317	sex	Diploid
ES	CC3	319	sex	Diploid
ES	CC3	320	sex	Diploid
TN	OUL	382	sex	Diploid
GR	COD	488	sex	Diploid
GR	COD	489	sex	Diploid
GR	COD	490	sex	Diploid
GR	COD	491	sex	Diploid
GR	COD	492	sex	Diploid
GR	COB	493	sex	Diploid
GR	COB	494	sex	Diploid
GR	COB	495	sex	Diploid
GR	COD	526	sex	Diploid
BE	DR1	616	asex	Triploid
PL	JAB	625	asex	Triploid
PL	JAB	626	asex	Triploid
PL	JAB	627	asex	Triploid
GR	COB	658	sex	Diploid
GR	COB	659	sex	Diploid
GR	COB	660	sex	Diploid
GR	COB	661	sex	Diploid
GR	COB	662	sex	Diploid
GR	COB	663	sex	Diploid
GR	COB	664	sex	Diploid
GR	COB	666	sex	Diploid
GR	COB	668	sex	Diploid
GR	COB	669	sex	Diploid
GR	COB	670	sex	Diploid
GR	COB	671	sex	Diploid
GR	COB	672	sex	Diploid
GR	COB	673	sex	Diploid
GR	COB	674	sex	Diploid
GR	COB	675	sex	Diploid
GR	COB	676	sex	Diploid
GR	COB	677	sex	Diploid
TN	OUL	719	sex	Diploid
TN	OUL	720	sex	Diploid
TN	OUL	721	sex	Diploid
TN	OUL	723	sex	Diploid
TN	OUL	724	sex	Diploid
TN	OUL	725	sex	Diploid
TN	OUL	726	sex	Diploid
TN	OUL	727	sex	Diploid
TN	OUL	728	sex	Diploid
TN	OUL	729	sex	Diploid
IT	SIF	8a	sex	Diploid
IT	SIF	10a	sex	Diploid
ES	CC4	15a	sex	Diploid

IT	SIF	16a	sex	Diploid
IT	SIF	18a	sex	Diploid
IT	SIF	25a	sex	Diploid
IT	CAV	26a	asex	Diploid
ES	CC4	31a	sex	Diploid
BE	DRN	32a	asex	?
GR	COE	36a	asex	Diploid
ES	CC4	39a	sex	Diploid
IT	SIF	42a	sex	Diploid
ES	CL2	43a	asex	Triploid
GR	COE	44a	asex	Diploid
UK	SEA	45a	asex	Diploid
ES	CC4	47a	sex	Diploid
IT	SIF	50a	sex	Diploid
UK	SEA	53a	asex	Diploid
ES	CC4	55a	sex	Diploid
IT	FOR	77a	asex	Diploid
IT	SIF	87a	sex	Diploid
FR	GAB	88a	asex	Diploid
DE	BUR	101a	asex	Triploid
ES	CL1	107a	asex	Triploid
ES	CL2	111a	asex	Triploid
UK	SEA	114a	asex	Triploid
ES	CC4	116a	asex	Triploid
ES	CL2	119a	asex	Triploid
ES	CC4	121a	asex	Diploid
UK	SEA	122a	asex	Triploid
GR	COD	125a	asex	Triploid
GR	COD	129a	asex	Triploid
UK	SEA	130a	asex	Triploid
DE	BUR	133a	asex	Triploid
DE	BUR	137a	asex	Triploid
ES	CL1	139a	asex	Triploid
GR	COD	141a	asex	Triploid
ES	CC4	143a	sex	Diploid
GR	COS	150a	asex	Triploid
UK	SEA	156a	asex	Triploid
FR	VLC	171a	asex	Triploid
HR	OMI	173a	asex	Triploid
ES	CC4	178a	asex	Triploid
GR	COD	180a	asex	Triploid
ES	CC4	182a	asex	Diploid
UK	SEA	185a	asex	Triploid
ES	CC4	186a	asex	Triploid
HR	OMI	187a	asex	Triploid
DE	BUR	188a	asex	Triploid
UK	BAM	190a	asex	Triploid
ES	CL2	202a	asex	Triploid
ES	CL2	203a	asex	Triploid
ES	CL2	214a	asex	Triploid
IT	FOR	215a	asex	Diploid
GR	COE	216a	asex	Triploid
ES	VA3	222a	asex	Triploid
DE	ZWE	223a	asex	Triploid

UK	SEA	229a	asex	Triploid
FR	GAB	230a	asex	Diploid
ES	CC4	233a	sex	Diploid
GR	COS	239a	asex	Triploid
DE	ZWE	241a	asex	Triploid
ES	CC4	246a	sex	Diploid
DE	ZWE	250a	asex	Triploid
UK	BAM	263a	asex	Triploid
IT	SIF	265a	sex	Diploid
IT	FOR	271a	asex	Diploid
ES	CL1	274a	asex	Triploid
ES	VA3	276a	asex	Triploid
BE	DRN	281a	asex	Triploid
ES	CC4	286a	asex	Diploid
UK	BAM	287a	asex	Triploid
IT	SIF	289a	sex	Diploid
GR	COE	295a	asex	Diploid
UK	SEA	296a	asex	Triploid
ES	CC4	108a	asex	Triploid
ES	CL1	123a	asex	Triploid
UK	SEA	124a	asex	Triploid
FR	FIO	266a	asex	Triploid
BE	DRN	311a	asex	Triploid
ES	CC5	1001a	asex	Triploid
ES	CC5	1002a	asex	Triploid
ES	CC5	1003a	asex	Triploid
ES	CC5	1004a	sex	Diploid
ES	CC5	1005a	sex	Diploid
ES	CC5	1006a	asex	Triploid
ES	CC5	1007a	asex	Diploid
ES	CC5	1008a	sex	Diploid
ES	CC5	1009a	asex	Triploid
ES	CC5	1011a	asex	Triploid
ES	CC5	1012a	asex	Triploid
ES	CC5	1014a	asex	Triploid
ES	CC5	1016a	asex	Triploid
ES	CC5	1018a	asex	Triploid
ES	CC5	1019a	asex	Triploid
ES	CC5	1020a	asex	Triploid
ES	CC5	1021a	asex	Triploid
ES	CC5	1023a	sex	Diploid
ES	CC5	1024a	asex	?
ES	CC5	1026a	asex	Triploid
ES	CC5	1028a	asex	Triploid
ES	CC5	1029a	asex	Triploid