Genetic structure of Austrian and Italian populations of *Limnocythere inopinata* (Crustacea, Ostracoda): a potential case of post-glacial parthenogenetic invader?

Valeria Rossi[1,*], Andrea Piotti[1], Walter Geiger[2], Giorgio Benassi[1] & Paolo Menozzi[1]

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

Several hypotheses were proposed to account for geographic parthenogenesis that is the disjointed distribution of a species' sexual and asexual forms. In the areas affected by glaciations the parthenogenetic forms would have had ecological success and hybridization and polyploidy would have been the driving forces in their success. *Limnocythere inopinata* is well-represented in Quaternary non-marine sequences and is useful for studying the evolution of geographic parthenogenesis. We analysed the genetic variability of twelve populations from sub alpine Italian lakes, Austrian lakes and a pond, in an area that was covered by ice during the Pleistocene. In all samples we found only parthenogenetic females. Using allozymes, we described at least 33 different clonal lineages in the total of 812 females. We found no evidence for polyploidy. Clonal diversity is relatively high and may reflect the genetic diversity of ancestral sexual metapopulations. In *L. inopinata*, geographical parthenogenesis seems the result of historical and ecological factors and is not linked to polyploidy and hybrid advantage.

Introduction

Geographic parthenogenesis appears as one more paradox in the theory of the evolution of sex. It is used to justify the separate spatial distribution of sexual and asexual forms in species that show both reproductive modes: asexual forms are relatively more common, often more successful, and more wide spread than the sexual forms from which they originated (Bell 1982, Suomalainen *et al*. 1987, Schwander & Crespi 2009). Several, different and non exclusive hypotheses have been proposed to account for geographical parthenogenesis (Bell 1982, Lynch 1984, Kearney 2005). According to the "post glacial invasion hypothesis", parthenogenetic forms would have had significant ecological success in the areas that were strongly affected by the Pleistocene glacial cycles. Parthenogenetic lineages would have spread from southern refu-

gia more efficiently than sexual ones. According to the "destabilizing hybridization hypothesis" (Lynch 1984), the maintenance of some kind of gene flow between sexual and parthenogenetic lineages resulted in the rapid displacement of one lineage by the other in areas of sympatry. More recently, Kearney (2005) and Lundmark and Saura (2006) stressed the importance of hybridization and polyploidy as driving forces in the ecological success of parthenogentic lineages (Kearney 2006, Lundmark 2005). The combination of different genomes often leads to high heterozygosity and polyploidy, and may create novel ecotypes with wider tolerance, ecological niche and distribution. Parthenogenesis, hybridization and polyploidy are intertwined phenomena that may contribute separately or together to the success of clonal taxa in new habitats such as those created after ice cover retreated at the end of the last glaciation.

Potential cases of post-glacial parthenogenesis include five main Ostracoda genera: *Eucypris*, *Ilyocypris*, *Heterocypris* (*Cyprinotus* in the American literature), *Limnocythere* and *Potamocypris* (Bell 1982, Turgeon & Hebert 1994, Horne & Martens 1999, Meisch 2000, Kearney 2005). Ostracods are one of the largest groups of Crustacea: non-marine taxa include about 2000 species and several lineages show both sexual and asexual reproduction (Martens *et al.* 2008). Due to calcified valves and sexual dimorphism, ostracods represent very important biostratigraphic indicators and can offer an excellent fossil record to test the historical scenario of geographic parthenogenesis (Griffiths & Butlin 1995). According to the "post glacial invasion hypothesis", after the last Pleistocene glaciation (20 000–15 000 yrs B.P.), parthenogenetic lineages spread from refugia in the circum-Mediterranean region and recolonised central and northern Europe (Late-glacial and Holocene). Data on the existence of sexual and asexual ostracod species in 34 Holocene sediment profiles from across Europe suggest that the advantage of parthenogenetic lineages over sexual ones lasted for a limited time (Griffiths & Butlin 1995). Asexual species would not have colonized water bodies earlier than sexual forms and, on average, sexual species had higher and more stable abundance than asexual species.

This suggests that the short-term cost of sexual reproduction was rapidly outweighed by its advantage in a changing environment. According to the "Holocene stability hypothesis", the current predominance of parthenogenetic populations would be the result of the relative climatic stability of the Holocene with parthenogenetic lineages outcompeting the sexual ones during the past 11 500 years (Dansgaard *et al.* 1993, Horne & Martens 1999).

Non-marine ostracods are among the most genetically variable parthenogens (Cywinska & Hebert 2002). The high incidence of transition from sexual to asexual reproduction that "froze" genetic diversity from sexual to asexual lineages, the interbreeding with sexual congeners or relatives, and much greater diversity in benthic than in planktonic asexuals are factors associated with high clonal diversity in *Cypridopsis vidua* and *Bradleystrandesia* (*Cypricercus* in the American literature) *reticulata* (Chaplin *et al.* 1994, Turgeon & Hebert 1994, Turgeon & Hebert 1995, Cywinska & Hebert 2002). These sources of diversity are particular relevant for geographic parthenogens *Heterocypris incongruens* and *Eucypris virens* (Rossi *et al.* 2006, Rossi *et al.* 2008). Intra and interspecific hybridization may result in the generation of polyploid lineages: both cytological and allozyme data indicate their relatively high frequencies in ostracods even if their occurrence in parthenogenetic lineages varied considerably (Tétart 1978, Little & Hebert 1994, Chaplin & Hebert 1997, Little & Hebert 1997, Turgeon & Hebert 1995, Little 2005). Data from North America indicate a higher incidence of polyploidy in ostracod species from higher latitudes but data from tropical habitats do not confirm this latitudinal pattern (Havel & Hebert 1989, Little & Hebert 1994, Rossi *et al.* 1998). When polyploid lineages were found in *H. incongruens* or in *E. virens*, their probably underestimated frequency (12%, in *H. incongruens* and 14% in *E. virens*) did not show any geographic or ecological cline (Rossi *et al.* 2006, Rossi *et al.* 2008). This suggests that polyploidy is not a major driving force in geographic parthenogenesis in these species. Nevertheless, the presence of polyploid lineages implies that hybridization may be considered as a route to parthenogenesis and a source of

genotypic diversity (Havel *et al*. 1990, Little & Hebert 1994, Chaplin *et al*. 1994, Turgeon & Hebert 1994, Turgeon & Hebert 1995, Chaplin & Hebert 1997, Cywinska & Hebert 2002, Rossi *et al*. 2006, Rossi *et al*. 2007, Rossi *et al*. 2008).

Limnocythere inopinata is a Holarctic species with a wide distribution in Europe where it shows a well-defined pattern of geographical parthenogenesis: sexual populations are known only from Macedonia and Turkey (Horne & Martens 1999). In the Western Paleartic rare males, that is 1 male for about 1000 females, are only occasionally found in northern and central European living populations (Meisch 2000). Unlike *H. incongruens* and *E. virens* which mainly colonised temporary water bodies, *L. inopinata* inhabits a wide variety of habitats including the shallow littoral of lakes. For this reason it is well-represented in Quaternary non-marine sequences and is potentially useful in the study of the evolution of geographic parthenogenesis. In support of the post-glacial invasion hypothesis it has been claimed that sexual populations of *L. inopinata* were present in Europe until the late Pleistocene, but only parthenogenetic populations recolonised the area after the last glacial retreat (Horne *et al*. 1998). In fact, sexual populations of this species seem to have been more widespread during the last Pleistocene interglacial and in the earliest Holocene than nowadays. Quaternary records of *L. inopinata* males include some from the late glacial and Holocene, suggesting that either sexual or parthenogenetic forms colonised the new habitats after ice retreated. Sexual populations have been migrating back and forth across Europe during warm climatic phases. Their widespread distribution during the last interglacial was due to strong climatic and environmental fluctuations in a period of less than 5000 years that probably favoured sexual lineages over parthenogenetic ones (Griffiths & Butlin 1995, Griffiths & Horne 1998). The current predominance of parthenogenetic populations would be the result of the relative climatic stability during the past 11 500 years (the "Holocene stability hypothesis", Dansgaard *et al*. 1993, Horne & Martens 1999).

Here we report the first data on genetic variability of *L. inopinata* from the Italian and the Austrian Alps, ice covered regions during the last glaciations. Our results can be compared with most data on genetic diversity and polyploidy in asexual non-marine ostracods that are based on allozyme markers.

Material and methods

Limnocythere inopinata is a benthic detritivorous species typically found in the littoral of many permanent and temporary habitats. Carapace shows great variation in size, shape and structure. Size of females has been reported between 0.50–0.70 mm; male carapace is longer (0.62–0.82 mm) and more elongate in the lateral view than the female one (Meisch 2000).

Samples were collected, either in spring 1995 or in late summer 1996, from the sediment–water interface of the littoral area of 12 water bodies from Italy and Austria during a survey of 21 sites (Rossi *et al*. 2004). The Italian populations of *L. inopinata* were from sub-Alpine lakes of glacial origin (Maggiore, Monate, Comabbio, Annone and Montorfano) and from lake Mantova inferiore (Ambrosetti & Barbanti 1992) (Fig. 1). The latter is a wetland area along the lower course of the Mincio river that originated from a catastrophic flood in 589 B.C. and the construction of dams in 1190. Water conductivity in these lakes ranged from 111 μS cm^{-1} (Monate) to 362 μS cm^{-1} (Mantova). The Austrian populations were taken from five lakes (Lieferinger Badeteich and Mondsee, located in the pre-Alpine zone of Upper Austria (Salzkammergut), Faakersee and Weizelsdorfer Badeteich (southern Austria, Carinthia) and Neusiedleresee (eastern Austria) and a pond, Oberer Strinkersee, located in the area of Seewinkel (eastern Austria, between Neusiedleresee and Hungarian border). The pond, although one of the most permanent in the area, dries out regularly in summer and is covered by ice in winter. Adult and late juvenile instars of *L. inopinata* were found in ice-covered water. Lieferinger Badeteich and Weizelsdorfer Badeteich are gravel pit lakes less than 50 years old. Mondsee lake formed about 18 000 years ago after the deglaciation of the surrounding area. Neusiedlersee is a shallow lake that occupies a tectonic depression in deep Pannonian sediments and originated at the end of Pleistocene
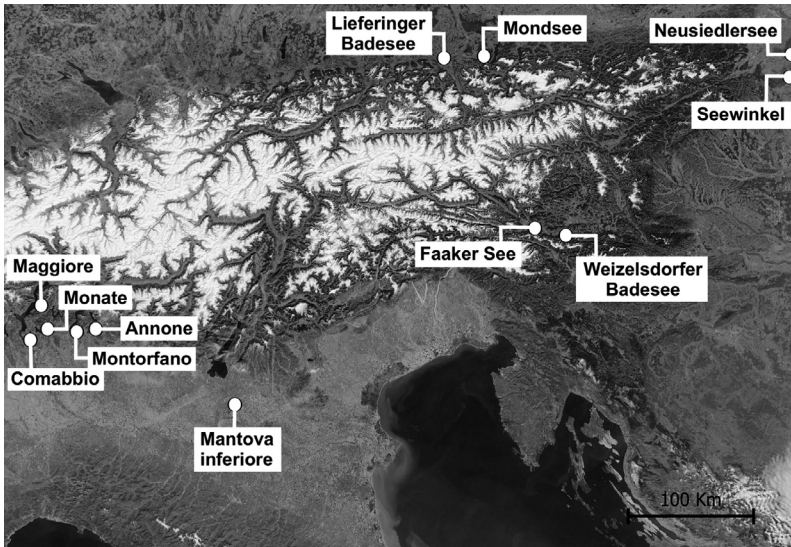
Fig. 1. Map of the sampling localities in northern Italy and Austria.

(12 000–14 000 yrs B.P.). Water conductivity ranged from 317 μS cm^{-1} (Mondsee) to 30 000 μS cm^{-1} (Oberer Strinkersee). In the eastern open part of lake Neusiedlersee and in the adjacent Seewinkel ponds, water has a characteristic high chloride content (up to 2160 mg l^{-1}). In these habitats three samples were collected in different seasons (late spring, late winter and late summer). All samples were transported alive and checked for species and gender under a Wild M8 stereomicroscope. The recognition of genders was based on carapace shape and, in dubious cases, on soft parts (sexual) organs (Meisch 2000). Organisms were maintained at 16 °C or frozen at –80 °C until the electrophoretic analysis.

The genetic structure of *L. inopinata* populations was determined by screening for variation at loci of eight enzymes: Got (glutamate-oxaloacetate transaminase E.C.2.6.1.1), Gpi (phosphoglucose isomerase E.C.5.3.1.9), Hk (hexokinase E.C.2.7.1.1), Idh (isocitrate dehydrogenase E.C.1.1.1.42), Ldh (lactate deydrogenase E.C.1.1.1.27), 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 was analysed by horizontal starch gel electrophoresis. Each allele was identified by its relative anodal migration according to increasing number and females from tested clonal lineages were used as internal standard on all analyses (Rossi *et al.*

2006). The presence of supernumerary bands and unbalanced staining activity were considered evidence of polyploidy (Little & Hebert 1994). The combined genotypes of the polymorphic loci were used to define the multilocus genotype of each individual.

Number of alleles per locus, number of multilocus genotypes, number of putative polyploidy genotypes were calculated for each population. The analysis of genetic diversity was carried out using the software GenoType/GenoDive: 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 (Meirmans & Van Tienderen 2004). Allele frequencies and heterozygosity were evaluated in samples of more than 20 individuals and with more than one multilocus genotype. 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 ver. 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 populations the analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) was

computed with GenoType/GenoDive (Meirmans & Van Tienderen 2004). A principal component analysis (PCA) for categorical data was performed to distinguish and discriminate populations according to the allele frequencies at polymorphic loci using the function "princomp" of the R statistical package (R Development Core Team 2008). The relationship between the two first principal components and geographic position was investigated using multiple regression. Furthermore, a 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. The phylogeography of genotypes was assessed using a Mantel test (R statistical package, R Development Core Team 2008).

Results

All populations consisted of females only: ovigerous females were present in all spring samples. Most individuals collected in the period from late September to November were juveniles and adults without eggs. A total of 812 individuals were sampled and screened (Table 1). Five enzymes (Got, Hk, Idh, Ldh, and 6Pgd) showed no activity when single individuals were assayed. Several individuals in different populations failed to show any activity or clear polymorphism at *Mpi* locus. For this reason only *Pgm* and *Gpi* loci and a total of 626 females, that showed activity either to *Pgm* or *Gpi*, were considered for describing the genetic structure of *L. inopinata*. The combined genotypes of these two loci defined the di-locus genotype (DLG) of a total of 488 females that showed activity both to *Pgm* and *Gpi* and were used for describing the clonal structure of *L. inopinata* populations.

A total of 3 and 7 alleles were detected at *Gpi* and *Pgm* loci, respectively. One allele at *Pgm* locus (allele 3) and one allele at *Gpi* locus (allele 2) were found in all populations. At *Pgm* locus, 3 alleles (1, 5 and 6) were exclusive of the eastern Austria populations from Neusiedlersee and Seewinkel. One allele at *Pgm* locus (allele 7) and one allele at *Gpi* locus (allele 1) were found only in the Italian population of Monate and the Austrian populations of Neusiedlersee and Seewinkel. PCA of the allele frequencies (93% explained variance by first two dimensions), showed that the populations' genetic structure was not associated with location (Fig. 2). The lack of geographic structure was confirmed by Mantel test ($p = 0.43$).

For the seven populations with more than one DLG and sample size larger than 20 females, mean heterozygosity by direct count ranged from 0.07 (Maggiore and Mondsee) to 0.46 (Annone) (Table 2). We found both excess and deficiency of heterozygotes. Only in the Monate

Table 1. For each sampling site (country: I = Italy, A = Austria), latitude (North), longitude (East), habitat (GL = glacial lake, AL = artificial lake, GPL = gravel pit lake, SL = shallow lake, P = pond), conductivity (EC), sample size (*n*), number of alleles per population at two polymorphic loci *Pgm* and *Gpi* (Na), number of di-locus genotypes (DLG) and Nei's genetic diversity index corrected for sample size (Div) are reported.

| Population	Lat. N	Long. E	Habitat	EC	*n*	Na	DLG	Div
1. Maggiore (I)	45°55′	08°37′	GL	147	30	4	4	0.30
2. Monate (I)	45°48′	08°40′	GL	111	75	6	10	0.75
3. Comabbio (I)	45°46′	08°40′	GL	160	90	4	5	0.58
4. Montorfano (I)	45°47'	09°08'	GL	215	19	6	4	0.42
5. Annone (I)	45°48′	09°20′	GL	242	51	4	4	0.59
6. Mantova (I)	45°09′	10°48′	AL	362	8	4	4	0.82
7. Faaker See (A)	46°34′	13°55′	GL	345	4	3	3	0.83
8. Mondsee (A)	47°50′	13°23′	GL	317	58	4	3	0.23
9. Lieferinger (A)	47°48′	13°03′	GPL	nd	51	2	1	0.00
10. Weizelsdorfer (A)	46°33′	14°13′	GPL	nd	9	4	1	0.00
11. Neusiedlersee (A)	47°46′	16°48′	SL	1000	181	9	14	0.65
12. Seewinkel (A)	47°48′	16°48′	P	30000	236	10	22	0.84
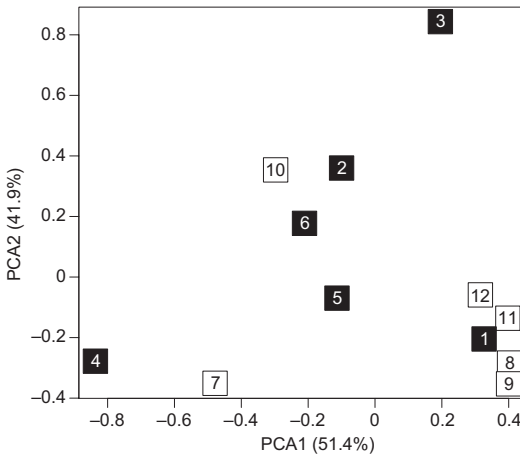
Fig. 2. Scatterplot of population scores resulting form a principal component analysis for allele frequency data. Black squares are Italian population and white squares are Austrian populations. The percentage of explained variance is reported for the first and second components. *See* Table 1 for population codes.

population, at both loci, and in the Neusiedlersee population, at *Pgm* locus, significant heterozygote excess was found. Loci were at significant linkage disequilibrium in the Annone and Seewinkel populations (Table 2). Allele frequencies were significantly different among populations considered together (χ^2 = 1239.48, df = 88, *p* < 0.001) and genetic differentiation was significant (AMOVA: phi-statistics = 0.063, *p* = 0.001).

Thirty-three DLGs were found. Genotype richness differed among populations and was not linked to sample size: we found only one DLG in Lieferinger Badesee (*n* = 51) and one in Weizelsdorfer Badesee (*n* = 9); in Faaker

See, where only four females were analysed, we found three DLGs (Table 1). The highest number of DLGs (22) was detected in the Seewinkel temporary pond. Nei's genetic diversity index corrected for sample size was between 0 and 0.84 and was not related to latitude ($F_{1,10}$ = 0.821, *p* = 0.386), longitude ($F_{1,10}$ = 0.003, *p* = 0.957) or water conductivity ($F_{1,8}$ = 1.618, *p* = 0.239). No supernumerary electrophoretic bands were found as evidence of polyploid genotypes.

The two most widespread DLGs (3322 and 3323) were found at 9 (75%) localities. The homozygote at both *Pgm* and *Gpi* loci (3322), was the most abundant and common clonal lineage, it was found in 69% of the sampled populations. Its frequency varied between 2.6% and 100% in the Italian and Austrian populations where it was found, and between 28.6% and 35.0% in Seewinkel and Neusiedlersee, respectively. In general, DLGs cannot be sorted into distinct groups by their allele similarity and no obvious spatial structure was observed in PCA. All populations were dominated by seven widespread clonal lineages whose frequencies were between 35% and 100%: 42% (14) of different DLGs were detected in more than one population while 19 DLGs (58%) were found only in one population (private DLGs). Among these rare clonal lineages 58% were from Seewinkel and 16% were from Neusiedlersee: their frequencies were between 0.9% and 1.8%. Private DLGs from these populations shared private alleles at the *Pgm* locus.

In Neusiedlersee and in Seewinkle the frequency of the three main DLGs (including more

Table 2. For polymorphic populations with *n* > 20, mean numbers of alleles per locus, mean heterozygosity by direct count (Ho) or expected according to Hardy-Weinberg equilibrium (He) are reported with standard deviation in parenthesses. Fixation index (F_{IS}) for significant heterozygote deficiency (F_{IS} > 0) or excess (F_{IS} < 0) for each polymorphic locus (*PGM* and *GPI*) is reported; ns indicates that the locus is in Hardy-Weinberg equilibrium. *P* values of genotypic linkage disequilibrium computations are reported (LD), nct = no contingency table.

	n	Alleles	Ho	He	PGM	GPI	LD
Maggiore	20.0 (1.0)	3.0 (1.5)	0.07 (.071)	0.17 (.029)	1.00	ns	0.21
Monate	51.5 (7.5)	3.0 (0.0)	0.62 (.090)	0.49 (.071)	−0.28	−0.28	0.29
Comabbio	69.5 (8.5)	2.0 (0.0)	0.12 (.107)	0.22 (.018)	0.93	ns	0.27
Annone	48.0 (3.0)	2.0 (0.0)	0.46 (.107)	0.40 (.077)	ns	ns	0.00
Mondsee	41.0 (7.0)	2.0 (1.0)	0.07 (.074)	0.07 (.070)	ns	−	nct
Neusiedlersee	135.5 (22.5)	4.5 (1.5)	0.30 (.246)	0.28 (.192)	−0.16	0.38	0.53
Seewinkel	170.0 (52.0)	5.0 (2.0)	0.30 (.104)	0.40 (.209)	0.33	ns	0.01

than 96% and 80% of scored females, respectively) varied significantly in seasonal samples ($\chi^2 = 27.667$, df = 10, $p < 0.01$ in Neusiedlerssee; $\chi^2 = 33.01$, df = 10, $p < 0.001$ in Seewinkle) and the seasonal distribution of the main DLGs varied in the two populations. The 3322 DLG showed the highest frequency in spring in Neusiedlersee and in late summer in Seewinkel ($\chi^2 = 7.627$, df = 2, $p < 0.01$). The second most frequent DLG, 3422, showed the highest frequency in winter in Neusiedlersee and in spring in Seewinkel ($\chi^2 = 16.745$, df = 2, $p < 0.001$). A third DLG, 4422, was detected in summer in Neusiedlersee while it was found in spring and winter in Seewinkle ($\chi^2 = 9.000$, df = 2, $p < 0.05$).

Discussion

Over the entire sampling area, we found only *Limnocythere inopinata* females. The recognition of females and the absence of males was based on carapace shape. We can exclude that the absence of males could be a seasonal phenomenon. Although caution is suggested by the low number of genetic markers, results like ours are in general seen as evidence of parthenogenesis. Sex ratio and genotypic frequency analysis have been considered valid criteria for breeding system assessment in ostracods (Havel *et al.* 1990, Little & Hebert 1994). We found no evidence for putative polyploid DLGs: this is a major reason to hypothesise that polyploidy was not a driving force in geographic parthenogenesis in *L. inopinata* (Kearney 2005). We cannot rule out that hybridization is involved in the ecological success of parthenogentic lineages of *L. inopinata*. However, it is generally observed that hybridization generates high heterozygosity (Little 2005, Schwander & Crespi 2009). Therefore, relatively low heterozygosity should suggest that parthenogenesis and genetic diversity are unlikely linked to hybridization in this species.

Apomixis, the reproductive parthenogentic mode described in several ostracod species, (*Cypridopsis vidua*, *Candonocypris novaezelandiae*, *Eucypris virens*, *Heterocypris incongruens* and *Cypris pubera*) is, in general, associated with heterozygote excess and linkage disequilibrium (Havel & Hebert 1989, Chaplin 1992,

Cywinska & Hebert 2002, Rossi *et al.* 2006, 2008). In *L. inopinata* we found low heterozygosity, Hardy-Weinberg and linkage equilibrium. Paradoxally, this result is not unusual in parthenogenetic ostracods and may have several explanations (Little & Hebert 1997, Rossi *et al.* 2006). A recent switch to asexual reproduction may be a cause of low heterozygosity and may explain the relatively low number of alleles we found in *L. inopinata*. In fact, a long-lived apomictic lineage is expected to increase its heterozygosity by accumulation of mutations that cannot be lost through segregation. In *E. virens*, the mean number of alleles per locus was not different between unisexual and sexual populations but, on average, heterozygosity was higher in the former (Rossi *et al.* 2008). Differences in heterozygosity between parthenogenetic and sexual populations were lower the more recent was the origin of asexual ones (Schön *et al.* 2000, Rossi *et al.* 2008). In *H. incongruens*, differences in heterozygosity between sexual and asexual populations were higher than in *H. barbara* in which the transition to asexuality was more recent (Rossi *et al.* 2003, 2006, 2007). The low heterozygosity we observed in *L. inopinata* might provide, as well as the fossil record, some evidence for recent (post glacial) adoption of asexuality. Finally, Hardy-Weinberg equilibrium might be due to the presence of null alleles coding for enzymatic proteins that show no or very low catalytic activity (Eanes 1987, Little & Hebert 1996). Hardy-Weinberg equilibrium observed in the obligate ancient asexual *Darwinula stevensoni* was attributed to the presence of null alleles (Rossi *et al.* 2004).

In *L. inopinata*, we detected 33 DLGs; clonal lineages' richness is probably underestimated due to the low number of markers used and the relative low sample sizes. Nevertheless, we consider that genotype diversity is comparable to the values observed in *H. incongruens* and *E. virens* that are among the most variable ostracod parthenogens species (Table 3). Genotype diversity in asexuals is generally considered a product of dynamic equilibrium between origins and stochastic or selective losses of clones (Butlin *et al.* 1999, Janko *et al.* 2008, Schwander & Crespi 2009). In *L. inopinata*, differences in clonal diversity among populations might be the result of

dynamic equilibrium between their origin, more or less recent from sexual or few asexual ancestors, and their habitat age. Both populations from the two less than 50 years old Austrian gravel pit lakes, Lieferinger Badeteich and Weizelsdorfer Badesee, where *L. inopinata* is the only ostracod species present, appeared to be monoclonal. Clonal lineages were different: the most common DLG was detected in Lieferinger while one of the rarest DLG was detected in Weizelsdorfer. This result may be due to the origin of these populations from a single or a few clonal females that, in case, impedes the establishment of further colonists by rapid population growth and monopolisation of resources (De Meester *et al.* 2002). In fact, in *C. vidua* (Havel & Hebert 1989, Little & Hebert 1994) and in *E. virens* (Rossi *et al.* 2008), habitats that are geographically isolated or that have recently been colonised, were monoclonal or showed very low clonal diversity. In our study, most populations with an intermediate number of DLGs were from glacial lakes. These populations were probably older than the ones from gravel-pit lakes and might originate from either asexual or sexual ancestors. In the first case, clonal richness could come from "stochastic differentiation" of a small number of ancestral asexual females through the accumulation of mutations. In the second case, the clonal richness could be the result of frozen genetic diversity from the pool of ancestral sexual lineages followed by the extinction of at least some of them. According to Danielopol *et al.* (1993), parthenogenetic lineages of *L. inopinata*, colonised the shallow part of the lake Mondsee as late as the postglacial Subatlantic climatic amelioration. Palaeoecological data on males occurrence in this lake are compatible with the origin of asexual lineages from sexual ones. Finally, the highest clonal richness we observed in *L. inopinata* populations from Neusiedlersee and Seewinkel might be linked to a relative more recent origin from a pool of sexual lineages. In fact, a fossil/historical occurrence of males has been reported from Neusiedlersee in short sections of cores dating approximately up to the 1930s (Löffler 1990). In these habitats, the relatively high number of clones might be the result of dynamic equilibrium between stochastic extinction and seasonal variation of conductivity and temperature that largely depends on the precipitation/evaporation cycle and allows for physical niche diversification. This maintains, at least in a short time scale, a relatively high number of specialised clonal lineages "frozen" along with adaptations for different niche partitions of the sexual population (Vrijenhoek 1984, Pound *et al.* 2004). Moreover, a transition from sexual to asexual reproduction due to spontaneous mutation that suppresses meiosis 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 males (Simon *et al.* 2003). This could explain the reports of rare *L. inopinata* males from France, Germany, Austria, Denmark, Hungary and in laboratory cultures from Seewinkel (Yin *et al.* 1999, Meish 2000). In *C. novaezelandiae* from south-eastern Australia the local displacement of sexual by conspecific clonal lineages is probably the result of recent and rapid expansion of parthenogenetic lineages (Chaplin 1992, 1993, Chaplin & Ayre 1989, 1997).

The wide spatial distribution of the most common clonal lineage does not seem to be due to the high dispersal and colonisation ability of *L. inopinata*. In fact, unlike most freshwa-

Table 3. Comparison of geographic parthenogenetic species. For each species: number of analysed populations (NP), number of scored individuals (Ns), number of polymorphic loci (NL), number of alleles (NA), number of multilocus genotypes observed (Go), Nei's genetic diversity index corrected for sample size (Div) and corrected Shannon-Wiener index (SWc) are reported.

Species	NP	Ns	NL	NA	Go	Div	SWc
L. inopinata	12	488	2	10	33	0.84	1.05
*H. incongruens**	47	3215	3	36	125	0.86	2.84
*E. virens***	21	694	3	31	75	0.82	1.20

*Rossi *et al.* 2006. **Rossi *et al.* 2008: populations from Extremadura, and Sicily where males were found are excluded from the present table.

ter ostracods, *L. inopinata* apparently does not produce resting eggs, although it has the ability to keep valves tightly closed or to rest in a torpid state (Geiger *et al*. 1998, Horne & Martens 1998). The most common DLG might be shared among geographically distant populations because its preferred habitat type is prevalent or because it has a broadly adapted genotype. Among the rarest clonal lineages, some DLGs were probably narrowly adapted to relatively high temperatures and conductivities of habitats from eastern Austria (Geiger *et al*. 1998). The apparent wide distribution and seemingly high ecological tolerance of *L. inopinata* is due to the existence of distinct clonal lineages representing a mixture of generalist and specialist strategies (Geiger *et al*. 1998).

The seasonal distribution of the main DLGs varied in Neusiedlersee and Seewinkel populations: clones that are rare in the lake may reach relatively high abundances in the pond. *L. inopinata* does not produce desiccation-resistant eggs and cannot survive long dry periods. These may wipe out a whole population and offer new space for genotypes from the source population. In the Seewinkel area, ponds are located in the proximity of permanent water bodies (adjacent lakes or other more permanent ponds) with high potentials for frequent colonisation over short distances (for instance water fowl) as predicted by a metapopulation model (Baltanás 1998, Cywinska & Hebert 2002, Frisch *et al*. 2007). Such dynamics could have played a role in geographic parthenogenesis because genetic bottlenecks and subsequent drift have stronger negative consequences for the fitness of sexual than for asexual forms (Haag & Ebert 2004).

In the geographic parthenogenesis of *L. inopinata*, the "post glacial invasion hypothesis" is not corroborated by the occurrence of sexual and asexual ostracod species in Holocene fossil sequences against a background of environmental change on a time scale of 5000 years (Chaplin *et al*. 1994, Griffiths & Butlin 1995). But we cannot exclude that, according to a model of geographically biased extinction (Schwander & Crespi 2009), sexual populations occurring in high-latitude or altitude regions might exhibit a higher extinction rate than those inhabiting regions that were less affected by glaciation. In

this case, extinctions should be the result of rapid loss of co-adapted gene complexes, rather than of the inability to adapt. On the other hand, the current predominance of parthenogenetic populations in western and central Europe might be the result of the relatively more stable predictable climatic situation of the Holocene, according to the "Holocene stability hypothesis" (Griffiths & Butlin 1995, Horne & Martens 1999, Martins *et al*. 2008).

In conclusion, we suggest that geographic parthenogenesis in *L. inopinata* reflects the influence of historical and ecological factors but seems not to be linked to polyploidy and hybrid advantage. Determination of the time scale over which parthenogenetic lineages can persist and provide an advantage over sexual ones is crucial, as both reproductive modes have various, but conflicting, benefits. Sexual lineages should be better competitors in long-lived habitats with geological stability, since they can adapt to changing environments with fine tuning (Bell 1992, Chaplin *et al*. 1994, Martens 1998). Parthenogenetic lineages are effective colonizers in short lived, erratic and/or predictably unstable habitats. We expect an asexual taxa to persist in a changeable environment when it is made up of an array of ecologically distinct clonal lineages (either generalist or specialist) with a unique, relatively narrow niche. Highly adapted clonal lineages (gene complexes), representing a particular combination of fitness-related life history traits, cannot be broken by recombination. As stressed by Martens (1998), it may be that time scales used were not appropriate for comparing colonising and adaptability efficiency and advantage of conspecific sexual and asexual lineages in changing or stable environments.

Further analyses and the joint use of molecular markers such as mitochondrial and nuclear DNA markers will greatly increase our ability to detect the actual, even hybrid, origin, age and phylogenetic relationship of different apomictic lineages of *L. inopinata* as well the role of rare males.

Acknowledgements

References

Ambrosetti, W. & Barbanti, I. 1992: Physical limnology in Italy: an historical review. — *Memorie dell'Istituto Italiano di Idrobiologia* 50: 37–59.

Baltanás, A. 1998: Ostracod populations as metapopulations. — In: Martens, K. (ed.), *Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine ostracods*: 229–241. Backhuys Publishers, Leiden, The Netherlands.

Bell, G. 1982: *The masterpiece of nature*. — Croom Helm, London.

Butlin, R. K., Schön, I. & Martens, K. 1999: Origin, age and diversity of clones. — *Journal of Evololutionary Biology* 12: 1020–1022.

Chaplin, J. A. 1992: Variation in the mode of reproduction among individuals of the ostracod *Candonocypris novaezelandiae*. — *Heredity* 68: 411–424.

Chaplin, J. A. 1993: The local displacement of a sexually reproducing ostracod by a conspecific parthenogen. — *Heredity* 71: 259–268.

Chaplin, J. A. & Ayre, D. J. 1989: Genetic evidence of variation in the contributions of sexual and asexual reproduction to populations of the freshwater ostracod *Candonocypris novaezelandiae*. — *Freshwater Biology* 22: 275–284.

Chaplin, J. A. & Ayre, D. J. 1997: Genetic evidence of widespread dispersal in a parthenogenetic freshwater ostracod. — *Heredity* 78: 57–67.

Chaplin, J. A. & Hebert, P. D. N. 1997: *Cyprinotus incongruens* (Ostracoda): an ancient asexual? — *Molecular Ecology* 6: 155–168.

Chaplin, J. A., Havel, J. E. & Hebert, P. D. N. 1994: Sex and ostracods. — *Trends in Ecology and Evolution* 9: 435–439.

Cywinska, A. & Hebert, P. D. N. 2002: Origins of clonal diversity in the hypervariable asexual ostracode *Cypridopsis vidua*. — *Journal of Evolutionary Biol*ogy 15: 134–145.

Danielopol, D. L., Handl, M. & Yin, Y. 1993: Benthic ostracods in the pre-alpine deep lake Monsee. Notes on their origin and distribution. — In: McKenzie, K. G. & Jones, P. J. (eds.), *Ostracoda in the earth and life sciences*: 465–480. A.A. Balkema, Rotterdam, The Netherlands.

Dansgaard, W., Johnsen, S. J., Clausen, H. B., Dahl-Jensen, D., Gundestrup, N. S., Hammer, C. U., Hvidberg, C. S., Steffensen, J. P., Sveinbjörnsdottir, A. E., Jouzel, J. & Bond, G. 1993: Evidence for general instability of past climate from a 250-kyr ice-core record. — *Nature* 364: 218–220.

De Meester, L., Gómez, A., Okamura, B. & Schwenk, K.

2002: The monopolization hypothesis and the dispersal-gene flow paradox in aquatic organisms. — *Acta Oecologica* 23: 121–135.

Eanes, W. F. 1987: Allozymes and fitness: evolution of a problem. — *Trends in Ecology and Evolution* 2: 44–48.

Excoffier, L., Smouse, P. E. & Quattro, J. M. 1992: Analysis of molecular variance inferred from metric distance among DNA haplotypes: application to human mitochondrial DNA restriction data. — *Genetics* 131: 479–491.

Frisch, D., Green, A. J. & Figuerola, J. 2007: High dispersal capacity of a broad spectrum of aquatic invertebrates via waterbirds. — *Aquatic Sciences* 69: 568–574.

Geiger, W., Otero, M. & Rossi, V. 1998: Clonal ecological diversity. — In: Martens, K. (ed.), *Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine ostracods*: 243–256. Backhuys Publishers, Leiden, The Netherlands.

Griffiths, H. I. & Butlin, R. K. 1995: A timescale for sex versus parthenogenesis: evidence from subfossil ostracods. — *Proceedings of the Royal Society B* 260: 65–71.

Griffiths, H. I. & Horne, D. J. 1998: Fossil distribution of reproductive modes in non-marine ostracods. — In: Martens, K. (ed.), *Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine ostracods*: 101–118. Backhuys Publishers, Leiden, The Netherlands.

Guo, S. & Thompson, E. T. 1992: Performing the exact test of Hardy-Weinberg proportion for multiple alleles. — *Biometrics* 48: 361–372.

Haag, C. R. & Ebert, D. 2004: A new hypothesis to explain geographic parthenogenesis. — *Annales Zoologici Fennici* 41: 539–544.

Havel, J. E. & Hebert, P. D. N. 1989: Apomictic parthenogenesis and genetic diversity in *Cypridopsis vidua* (Ostracoda, Cyprididae). — *Heredity* 62: 383–392.

Havel, J. E., Hebert, P. D. N. & Delorme, L. D. 1990: Genotypic diversity of asexual Ostracoda from a low Arctic site. — *Journal of Evolutionary Biology* 3: 391–410.

Horne, D. J., Baltanás, A. & Paris, G. 1998: Geographical distribution of reproductive modes in living non-marine ostracods. — In: Martens, K. (ed.), *Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine ostracods*: 77–99. Backhuys Publishers, Leiden, The Netherlands.

Horne, D. J. & Martens, K. 1998: An assessment of the importance of resting eggs for the evolutionary success of Mesozoic non-marine cypridoidean Ostracoda (Crustacea). — *Archiv für Hydrobiologie* 52: 549–561.

Horne, D. J. & Martens, K. 1999: Geographical parthenogenesis in European non-marine ostracods: post-glacial invasion or Holocene stability? — *Hydrobiologia* 391: 1–7.

Kearney, M. 2005: Hybridization, glaciation and geographic parthenogenesis. — *Trends in Ecology and Evolution* 20: 495–502.

Kearney, M. 2006: Response to Lundmark: polyploidization, hybridization and geographical parthenogenesis. — *Trends in Ecology and Evolution* 21: 10.

Janko, K., Drozd, P., Flegr, J. & Pannell, J. R. 2008: Clonal turnover versus clonal decay: a null model for observed

patterns of asexual longevity, diversity and distribution. — *Evolution* 62: 1264–1270.

Little, T. J. 2005: Genetic diversity and polyploidy in the cosmopolitan asexual ostracod *Cypris pubera*. — *Journal of Plankton Research* 27: 1287–1293.

Little, T. J. & Hebert, P. D. N. 1994: Abundant asexuality in tropical freshwater ostracodes. — *Heredity* 73: 549–555.

Little, T. J. & Hebert, P. D. N. 1996: Ancient asexuals: scandal or artifact? — *Trends in Ecology and Evolution* 11: 296.

Little, T. J. & Hebert, P. D. N. 1997: Clonal diversity in high arctic ostracodes. — *Journal of Evolutionary Biology* 10: 233–252.

Löffler, H. 1990: Paleolimnology of Neusiedlrersee, Austia. I. The succession of Ostracods. — *Hydrobiologia* 214: 229–238.

Lundmark, M. 2005: Polyploidization, hybridization and geographical parthenogenesis. — *Trends in Ecology and Evolution* 21: 9.

Lundmark, M. & Saura, A. 2006: Asexuality alone does not explain the success of clonal forms in insects with geographical parthenogenesis. — *Hereditas* 143: 23–32.

Lynch, M. 1984. Destabilizing hybridization, general-purpose genotype and geographic parthenogenesis. — *Quarterly Review of Biology* 59: 257–290.

Martens, K. 1998: Sex and ostracods: a new synthesis. — In: Martens, K. (ed.), *Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine ostracods*: 295–321. Backhuys Publishers, Leiden, The Netherlands.

Martens, K., Schön, I., Meisch, C. & Horne, D. J. 2008: Global diversity of Ostracods (Ostracoda, Crustacea) in freshwater. — *Hydrobiologia* 595: 185–193.

Martins, M. J. F., Vandekerkhove, J. & Namiotko, T. 2008: Environmental stability and the distribution of the sexes: insights from life history experiments with the geographic parthenogen *Eucypris virens* (Crustacea: Ostracoda). — *Oikos* 117: 829–836.

Meirmans, P. G. & Van Tienderen, P. H. 2004: GENOTYPE and GENODYVE: two programs for the analysis of genetic diversity of asexual organisms. — *Molecular Ecology Notes* 4: 792–794.

Meisch, C. 2000: *Freshwater Ostracoda of western and central Europe*. — Süßwasserfauna von Mitteleuropa 8/3, Spektrum Akademischer Verlag, Heidelberg, Berlin.

Pound, G. E., Cox, S. J. & Doncaster, C. P. 2004: The accumulation of deleterious mutations within the frozen niche variation hypothesis. — *Journal of Evolutionary Biology* 17: 651–662.

R Development Core Team 2008: R: *A language and Environment for Statistical Computing*. — R Foundation for Statistical Computing, Vienna, Austria.

Raymond, M. & Rousset, F. 1995: GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. — *Journal of Heredity* 86: 248–249.

Rossi, V., Schön, I., Butlin, R. K. & Menozzi, P. 1998: Clonal genetic diversity. — In: Martens, K. (ed.), *Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine ostracods*: 257–274. Backhuys Publishers, Leiden, The Netherlands.

Rossi, V., Gandolfi, A., Gentile, G., Geiger, W. & Menozzi,

P. 2004: Low genetic variability in the ancient asexual ostracod *Darwinula stevensoni*. — *Italian Journal of Zoology* 71: 135–142.

Rossi, V., Benassi, G., Leonardi, S., Piotti, A. & Menozzi, P. 2006: Clonal diversity of *Heterocypris incongruens* (Crustacea, Ostracoda) in northern Italian ricefields. — *Archiv für Hydrobiologie* 166: 225–240.

Rossi, V., Gandolfi, A., Baraldi, F., Bellavere, C. & Menozzi, P. 2007: Phylogenetic relationships of coexisting *Heterocypris* (Crustacea, Ostracoda) lineages with different reproductive modes from Lampedusa Island (Italy). — *Molecular Phylogenetics and Evolution* 44: 1273–1283.

Rossi, V., Piotti, A., Baltanás, A., Benassi, G. & Menozzi, P. 2008: Genetic diversity and mixed reproduction in *Eucypris virens* (Crustacea: Ostracoda). — *Fundamental and Applied Limnology* 172: 147–159.

Rossi, V., Tondelli, B., Gandolfi, A., Bellavere, C., McKenzie, K. G. & Menozzi, P. 2003: *Heterocypris* (Crustacea, Ostracoda) from the Isole Pelagie (Sicily, Italy): population genetics. — *Italian Journal of Zoology* 70: 101–108.

Schön, I., Gandolfi, A., Di Masso, E., Rossi, V., Griffiths, H. I., Martens, K. & Butlin, R. K. 2000: Persistence of asexuality through mixed reproduction in *Eucypris virens* (Crustacea, Ostracoda). — *Heredity* 84: 161–169.

Schwander, T. & Crespi, B. J. 2009: Twigs on the tree of life? Neutral and selective models for integrating macroevolutionary patterns with microevolutionary processes in the analysis of asexuality. — *Molecular Ecology* 18: 28–42.

Simon, J. C., Delmotte, F., Rispe, C. & Crease, T. 2003: Phylogenetic relationships between parthenogens and their sexual relatives: the possible routes to parthenogenesis in animals. — *Biological Journal of the Linnean Society* 79: 151–163.

Suomalainen, E., Saura, A. & Lokki, J. 1987: *Cytology and evolution in parthenogenesis*. — CRC Press, Boca Raton, Florida.

Tétart, J. 1978: Les garnitures chromosomiques des Ostracodes d'eau douce. — *Travaux du Laboratorie d'Hydrobiologie et de Pesciculture de l'Université de Grenoble* 69–70: 113–140.

Turgeon, J. & Hebert, P. D. N. 1994: Evolutionary interaction between sexual and all-females taxa of *Cyprinotus* (Ostracoda: Cyprididae). — *Evolution* 48: 1855–1865.

Turgeon, J. & Hebert, P. D. N. 1995: Genetic characterization of breeding systems, ploidy levels and species boundaries in *Cypricercus* (Ostracoda). — *Heredity* 75: 561–570.

Vrijenhoek, R. C. 1984: Ecological differentiation among clones: the frozen niche variation model. — In: Wöhrmann, K. & Loeschcke, V. (eds.), *Population biology and evolution*: 217–231. Springer-Verlag, Heidelberg.

Weir, B. S. & Cockerham, C. C. 1984: Estimating *F*-statistics for the analysis of population structure. — *Evolution* 38: 1358–1370.

Yin, Y., Geiger, W. & Martens, K. 1999: Effects of genotype and environment on phenotypic variability in *Limnocythere inopinata* (Cristacea: Ostracoda). — *Hydrobiologia* 400: 85–114.