

# Sex ratio, reproductive mode and genetic diversity in *Triops cancriformis*

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## SUMMARY

1. Aquatic invertebrates display a wide array of alternative reproductive modes from apomixis to hermaphroditism and cyclical parthenogenesis. These have important effects on genetic diversity and population structure. Populations of the 'living fossil' *Triops cancriformis* display a range of sex ratios, and various reproductive modes are thought to underlie this variation. Using sex ratio information and histological analyses European populations have been inferred to be gonochoric (with separate males and females), selfing hermaphroditic and androdioecious, a rare reproductive mode in which selfing hermaphrodites coexist with variable proportions of males. In addition, some populations have been described as meiotic parthenogens.

2. Here we use population genetic analysis using microsatellite loci in populations with a range of sex ratios including a gonochoric population, and marker segregation patterns in heterozygote individuals reared in isolation, to clarify the reproductive mode in this species.

3. Our data show that populations in general have very low levels of genetic diversity. Non-gonochoric populations show lower genetic diversity, more heterozygote deficiencies, higher inbreeding coefficients and stronger linkage disequilibria than the gonochoric population. The maintenance of some heterozygosity in populations is consistent with some male influence in *T. cancriformis* populations, as would be expected from an androdioecious reproductive system. Results of marker segregation in eggs produced in isolation from non-gonochoric populations indicate that meiosis occurs and are consistent with two reproductive modes: selfing hermaphroditism and a type of ameiotic parthenogenesis.

4. Overall, our data indicate that androdioecy and selfing hermaphroditism are the most likely reproductive modes of non-gonochoric European *Triops* populations. *Triops* populations are strongly structured, suggesting high genetic drift and low levels of gene flow.

*Keywords:* androdioecy, automixis, linkage disequilibrium, microsatellites, parthenogenesis

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## Introduction

Most aquatic invertebrates of temporary waters survive adverse environmental periods through some form of dormant stage (Hairston, 1996; Caceres, 1997; Brendonck, De Meester & Hairston, 1998). In contrast to this relatively uniform feature of their life cycles, these organisms display a wide diversity of alternative reproductive modes, departing from the often ancestral gonochoric reproductive mode (separate males and females) exhibited by most copepods and anostracans. These range from apomictic parthenogenesis (e.g. bdelloid rotifers, some cladocerans, some ostracodes (Butlin, Schon & Martens, 1998; Taylor, Crease & Brown, 1999; Welch, Welch & Meselson, 2004), automictic parthenogenesis (e.g. *Artemia parthenogenetica* Bowen and Sterling) (Browne, 1992), cyclical parthenogenesis (most cladocerans and monogonont rotifers (Gilbert, 1993; Taylor *et al.*, 1999), to hermaphrodite or androdioecious (e.g. clam shrimps *Eulimnadia*; Weeks *et al.*, 2008). Transitions in reproductive mode and mating strategy (e.g. from outcrossing to selfing or from sexual to asexual reproduction) have occurred repeatedly in several groups (Sassaman, 1995; Butlin *et al.*, 1998; Taylor *et al.*, 1999), possibly as adaptive responses to environmental challenges: uniparental modes of reproduction (hermaphroditism or parthenogenesis) can result in more efficient colonisation of new habitats (Pannell & Barrett, 1998). Efficient colonisation is likely to be favoured when habitats are recent or are fragmented and short lived, promoting the occurrence of metapopulations (Obbard, Harris & Pannell, 2006; Pannell & Dorken, 2006) as is often the case in temporary ponds (Haag *et al.*, 2002). In turn, the mode of reproduction has important consequences for genetic diversity, population structure, genomic structure and the evolutionary potential of organisms (Barrett, 1998; Holsinger, 2000; Charlesworth & Wright, 2001). Therefore, a better understanding of the reproductive mode of freshwater organisms is crucial for interpreting many aspects of their ecology, evolution and, additionally, can inform management strategies.

The Notostraca is a small order of branchiopod crustaceans showing an extremely conserved morphology, with fossils from 300 million years ago being morphologically identical to extant taxa (Tasch, 1963), but a number of different reproductive modes

(Sassaman, 1991). The dozen or so described species inhabit ephemeral water bodies and produce resistant diapausing cysts that form egg banks in sediments and are also the dispersal stage (Thiery, 1991, 1997). The ancestral reproductive mode, present in several species seems to be gonochorism (Pannell, 2002; Zierold, Hänfling & Gómez, 2007), with equal sex ratio and copulation being necessary in order to lay viable eggs (Korn *et al.*, 2006). In contrast, certain populations of several extant species are characterised by an ability of individuals to breed in isolation, a lack of males and the existence of ovotestis (Zaffagnini & Trentini, 1980) suggesting the occurrence of hermaphroditism (Longhurst, 1955a; Sassaman, 1991). A third reproductive mode, androdioecy (AD), has also been described in Notostracans. Androdioecy is an extremely rare mixed reproductive strategy in animals, in which breeding populations consist of both self-fertile hermaphrodites and males, the latter often found in very low proportion (Charlesworth, 1984; Pannell, 2002; Weeks, Benvenuto & Reed, 2006). Unlike the case in plants (Pannell, 2002), hermaphrodites are incapable of cross-fertilising one another, therefore outcrossing depends exclusively on the presence of males in the population.

The proportion of males in androdioecious populations is therefore predicted to have marked effects on population genetic diversity and fitness. Self-fertilisation leads to very high rates of intra-individual homozygosity and high linkage disequilibrium resulting in a quasi-clonal population structure (Mackiewicz *et al.*, 2006b). As a consequence, self-fertilisation is expected to lead to inbreeding depression in the resulting offspring (Weeks, Marcus & Crosser, 1999; Weeks *et al.*, 2000; Weeks, 2004), unless effective purging takes place due to repeated selfing (Dolgin *et al.*, 2007). The presence of males in both killifish, *Kryptolebias marmoratus* (Huber), and clam shrimp, *Eulimnadia texana* (Packard), populations has a large impact on individual heterozygosity, strongly reducing population level inbreeding (Weeks & Zucker, 1999; Mackiewicz *et al.*, 2006a; but see Dolgin *et al.*, 2007). On the other hand, the presence of males will increase effective population size, predictably decreasing drift and helping to maintain genetic diversity, as shown in American *Triops* populations (Sassaman, Simovich & Fugate, 1997).

The tadpole shrimp *Triops cancriformis* Bosch, 1801 (Crustacea: Branchiopoda: Notostraca) inhabits rice

fields, rain pools, fishery ponds and other freshwater, temporary waterbodies throughout Europe. North European populations are closely related, whereas southern populations are more genetically differentiated (Korn *et al.*, 2006; Zierold *et al.*, 2007). All three reproductive modes described for the Notostraca and a range of sex ratios have been found in this species (Zaffagnini & Trentini, 1980; Sassaman, 1991; Zierold *et al.*, 2007). Iberian populations are considered gonochoric (1 : 1 sex ratio) and Central and Northern European populations, with no or a low proportion of males, are considered hermaphrodite and androdioecious (Zierold *et al.*, 2007). The reproductive strategy in some Central European and Italian populations, however, remains controversial. Females from a German *T. cancriformis* population with a low proportion of males apparently lacked testicular tissue (Engelmann, Hahn & Hoheisel, 1997), suggesting parthenogenesis or a mixture of gonochoric and hermaphroditic reproduction. In addition, some Italian populations have also been claimed to be meiotic parthenogens (Trentini & Scanabissi, 1979), with males absent from populations. However, the presence of testicular lobes (Zaffagnini & Trentini, 1980), meiosis (Scanabissi & Trentini, 1979) and very reduced levels of genetic diversity (Cesari *et al.*, 2004; Mantovani *et al.*, 2008) are also consistent with selfing hermaphroditism, as in other *Triops* species (Sassaman, 1991). In addition, the use of sex ratio and histological analysis to infer reproductive mode has been criticized (Sassaman, 1991). So, far, no experimental analysis of genetic inheritance on this species, which would give conclusive results as to the form of reproduction, has been carried out to shed light into the reproductive mode of populations. The understanding of the mode of reproduction in this species is more urgent than ever, as the species is considered of conservation concern or even extinct in several European countries mainly due to habitat loss and pollution (Hughes, 1997; Damgaard & Olesen, 1998; Boix i Masafret, 2002; Eder & Hödl, 2002). In the U.K., there is an action plan in place for the conservation of this species (<http://www.ukbap.org.uk/UKPlans.aspx?ID=617>).

In this study, we used population scoring for eight microsatellite loci (three of them newly isolated) and breeding experiments to understand further the reproductive mode of this species. In particular we tested: (i) the effect of sex ratio on the genetic diversity, inbreeding coefficients and linkage disequi-

librium in *T. cancriformis* populations. We described the genetic diversity and level of inbreeding of *T. cancriformis* populations with a range of sex ratios to see if population genetic diversity and structure shed light on the reproductive mode of populations. (ii) Microsatellite inheritance patterns in the offspring of isolated females. We designed isolation experiments to assess reproduction in isolation versus in groups, and genotyped the offspring of isolated individuals to understand the reproduction mechanism of different *Triops* populations.

## Methods

### Samples

As part of a wider project, we sampled 29 European *T. cancriformis* populations between 2000 and 2006 (see Zierold *et al.*, 2007 for details). Note that the samples analysed here do not include the former subspecies *T. cancriformis mauritanicus*, which occurs in most of Spain and N Africa and is now regarded as a different species, *Triops mauritanicus* (Korn *et al.*, 2006; Zierold *et al.*, 2007). The populations sampled showed a range of sex ratios, including populations with no males (Neuburg), a low proportion of males (Koenigswartha) and one population with an equal proportion of males and females, which had been inferred to be gonochoric (Espolla). For population analyses, samples included specimens collected from natural populations using a hand net (5 mm mesh), and sediment containing diapausing cysts from dry ponds where the species was known to occur. Individuals reared directly from sediment samples were considered 'field collected' as they existed as embryos in the field. When specimens were needed for genetic analyses or reproduction experiments, sediment was placed at the bottom of glass tanks and distilled water added to induce the hatching of cysts. All specimens used in the genetic analyses were sexed (males were identified by their lack of a modified 11th thoracic leg for egg carrying) and this information was added to the sex ratio information available for the studied populations.

### Microsatellite development

Genomic DNA from one *T. cancriformis* individual from the Koenigswartha population was obtained by a standard phenol-chloroform technique (Sambrook

& Russell, 2001) and a partial genomic library was obtained by digesting the DNA extract with the enzyme *Sau3AI* (Promega, Madison, WI, U.S.A.). Following electrophoresis on a 2% agarose minigel, the 300–600 bp size region was excised and the digested DNA recovered by centrifugation. To enrich the partial genomic library of *T. cancriformis* for microsatellites, we hybridized the size-selected DNA fragments to biotinylated (TG)<sub>22</sub> and (GA)<sub>22</sub> microsatellite-motif probes, and then recovered these molecule-genomic hybrids with avidine following the protocol of Hammond *et al.* (1998). The amplified microsatellite-enriched DNA was ligated into PGEM<sup>®</sup>-T easy vectors (Promega) and transformed into DH5 $\alpha$ <sup>TM</sup> Competent Cells (Invitrogen, Carlsbad, CA, U.S.A.).

A total of 308 successfully transformed colonies were then screened for TG and GA microsatellites (separately) using the PIMA approach of Lunt, Hutchinson & Carvalho (1999). The 34 colonies yielding extra bands were sequenced on a CEQ<sup>TM</sup> 8000 Genome Analysis Suite System (Beckman-Coulter<sup>TM</sup>, Fullerton, CA, U.S.A.) using a sequencing kit (GenomeLab<sup>TM</sup> Dye Terminator Cycle Sequencing with Quick Start Kit, Beckman-Coulter<sup>TM</sup>, Fullerton, CA, U.S.A.). Fourteen of the sequenced colonies presented one or two microsatellites. For those sequences containing microsatellites, primers were designed using OLIGO v. 6.67 (Molecular Biology Insights, West Cascade, CO, U.S.A.). Three of the microsatellite loci designed could be optimized and shown to be polymorphic in our dataset (see Table 1).

#### DNA extraction and microsatellite typing

Tissues from field collected samples (see above) were stored in ethanol and total genomic DNA was isolated using CTAB and commercial DNA extraction kits

(Invisorb Spin Forensic Kit, Invitek, Berlin, Germany; PureGene Kit, Qiagen, Valencia, CA, U.S.A.). Egg bearing individuals from the isolation experiments were snap frozen and their eggs removed before they were fixed in ethanol. DNA extraction from eggs was carried out using the Hotshot method (Montero-Pau, Gómez & Muñoz, 2008).

Ten natural populations from which 10 or more individuals could be obtained were screened using a set of five microsatellite loci developed by Cesari *et al.* (2004). We additionally typed the four populations used in the reproduction experiments (see below) with the three newly developed microsatellite primers to maximise the chances of obtaining heterozygote individuals (see Table 1). Microsatellite amplifications were performed in 15  $\mu$ L final volume containing 2.5  $\mu$ L template DNA (diluted 1 : 50 from the Hotshot extraction), 2.5 mM MgCl<sub>2</sub>, 200  $\mu$ M of each nucleotide, 300  $\mu$ M of each primer, 0.02 U of *Taq* DNA polymerase (Bioline, London, U.K.) and 1x NH<sub>4</sub> PCR Buffer (Bioline). The following cycling conditions were used: 3 min denaturing at 94 °C, (15 s at 94 °C, 20 s at the appropriate annealing temperature, 40 s at 72 °C)  $\times$  34 and 10 min final extension at 72 °C (the final extension step was not performed if stutter peaks appeared in the microsatellite typing). The amplified fragments were separated in a CEQ<sup>TM</sup> 8000 Genome Analysis System (Beckman-Coulter<sup>TM</sup>) and all peaks were scored using the software provided by the manufacturer.

#### Analysis of population structure and genetic diversity

Observed and expected heterozygosity were computed using GenAIE<sub>x</sub> (Peakall & Smouse, 2006). An unbiased estimate of allelic diversity (allelic richness, *A*) was obtained using the rarefaction method implemented in FSTAT 2.9 (Goudet, 1995). Note that estimates of *A* are

**Table 1** Microsatellite loci for *Triops cancriformis* developed in this study. GenBank accession numbers are given under locus names. The repeat motifs and primer sequences are given (F: forward primer, R: reverse primer).  $T_A$  is the annealing temperature used in the PCR. Size range and number of alleles per locus (*A*) are also given

Locus name & Accession number	Repeat motif	Primer sequences (5' to 3')	$T_A$ (°C)	Size range (bp)	<i>A</i>
tcGA-10p2 EU744335	(GA) <sub>2</sub> GC(GA) <sub>4</sub> GC(GA) <sub>3</sub>	F: ATGGATTGGGCTGCTGAG R: GGCATGTTGATTACACAAGA	54	277–287	6
tcCA-6p5 EU744333	CAGA(CA) <sub>5</sub>	F: CGCTTATGAAACACATGGACC R: CCCAAATTCCTAGACGAAAC	52	154–162	5
tcGA-8p8 EU744334	(GA) <sub>2</sub> A <sub>2</sub> (GA) <sub>6</sub>	F: CAACAAGTCTGCGAGATACGAC R: CCCGAGTGGTGAAGCAAC	52	192–221	10

based on only the five loci which were scored for all populations. Linkage disequilibrium and population tests for Hardy–Weinberg equilibrium (HWE, with H1: heterozygote deficiency) were carried out with the program Genepop, v. 4.0 (Rousset, 2008). Estimates of population specific  $F_{IS}$  values and global and pairwise multilocus and  $F_{ST}$  values (Weir & Cockerham, 1984) were computed with FSTAT v. 2.9 (Goudet, 1995). Significance of  $F_{ST}$  pairwise values was tested using randomisation tests (10 000 permutations) permuting genotypes among samples thus not assuming HWE. Randomisation procedures (10 000 permutations) were also used to test significant differences in genetic diversity among samples from populations with a low proportion of males and from populations in which no males were found.

We tested for correlations between genetic ( $F_{ST}$ ) and geographic distance ( $\log_{10}$  of distance between populations, measured using Google Earth) including and excluding the Espolla population. We used the program IBDWS v. 3.15 (Jensen, Bohonak & Kelley, 2005) to perform Mantel tests (1000 randomisations), to estimate the slope and intercept of the correlation using reduced major axis regression analyses, and to plot the relationship between genetic and log (geographic distances).

Principal component analysis (PCA) was used to produce a visual representation of differentiation among the samples using the program PCA-GEN (available from J. Goudet at <http://www2.unil.ch/popgen/softwares/pcagen.htm>).

Partial Bayesian cluster analysis of pre-defined groupings (samples) was performed using BAPS 5.1 (Corander, Waldmann & Sillanpaa, 2003; Corander & Marttinen, 2006), the aim of which is to identify the optimum number of partitions ( $K$ ) among groups of samples. The analysis was run using maximum  $K$  settings from 1 to 10 repeating the analysis five times at each value. The overall posterior probability for each value of  $K$  was calculated from the log-likelihood values across all runs.

#### Reproduction experiments

We carried out reproduction experiments in seven populations for which enough hatchlings were obtained (see Table 4). Our objective was to test for hermaphroditic or parthenogenetic reproduction in isolated individuals. Two experimental set-ups were used: isolation experiments and, as a control for adequate conditions for reproduction, a mixed-rearing experimental set-up (for sample sizes for each experiment see Table 4). Due to limitation in the amount of sediment available for each population and the number of hatchlings obtained, both experimental set-ups could only be completed in four populations representing a range of sex ratios: ESP (equal sex ratio), KOE27 and KOE12 (low proportion of males), and KOE21 and NEU (no males) (see Table 2). Several mixed experiments could be completed for KOE12, NEU and GOD. Due to the nature of the experiment, it was difficult to replicate identical conditions in

**Table 2** The proportion of males and genetic variation in 10 *Triops cancriformis* population samples based on five microsatellite loci [for more population details see Zierold *et al.* (2007)]. The populations are ordered according to a decreasing proportion of males (in parenthesis, sample size for calculating proportion of males).  $N$ , sample size for microsatellite analysis; % polymorphic loci (0.99 criterion);  $A$ , allelic richness (standard deviation);  $H_e$ , expected heterozygosity given the observed allele frequencies and assuming a randomly mating gonochoric population in Hardy–Weinberg equilibrium;  $H_o$ , observed heterozygosity, and  $F_{IS}$  are also given

Population	Code	Male proportion ( $n$ )†	$N$	% polymorphic loci	$A$ (SD)	$H_e$	$H_o$	$F_{IS}$
Espolla, Spain	ESP	0.529 (121)	66	87.5	1.65 (0.47)	0.21	0.15	0.287**
Königswartha, Germany	KOE27	0.102 (137)	52	87.5	2.29 (0.82)	0.36	0.04	0.883**
Königswartha, Germany	KOE12	0.095 (105)	67	62.5	1.49 (0.61)	0.14	0.07	0.456**
Lacoma, Germany	LAC1	0.067 (15)	13	20	1.2 (0.44)	0.04	0.05	−0.091
Lacoma, Germany	LAC2	0.067 (15)	15	0	1 (0.0)	0	0	−
Kaiserlacke, Austria	KAI	0.063 (16)	17	40	1.38 (0.51)	0.11	0	1.000**
Königswartha, Germany	KOE28	0.020 (50)	10	20	1.4 (0.89)	0.05	0.02	0.654**
Neuburg, Germany	NEU	0.000 (102)	51	75	1.38 (0.59)	0.07	0.03	0.508*
Godshill pond, U.K.	GOD	0.000 (25)	18	40	1.40 (0.55)	0.16	0.04	0.539*
Königswartha, Germany	KOE21	0.000 (19)	15	40	1.4 (0.5)	0.20	0.11	0.482**

The significance of  $F_{IS}$  is indicated as \*\*highly significant,  $P < 0.001$ , \*significant,  $P < 0.05$ . Data for populations ESP, KOE12, KOE27 and NEU was computed for eight loci.

†Data compiled from Zierold *et al.* (2007) and Zierold (2006) and this work.

isolated and mixed rearing individuals, although we kept them as similar as possible. For the isolation experiments, individual hatchlings were isolated in plastic containers with c. 200 mL of mineral water and sand in the bottom (they were a few days old, and therefore virgin). In the mixed-rearing experiments, sediment from a single pond was placed at the bottom of 20 L aquaria, the tank was half-filled with distilled water and the individual hatchlings were reared together. We did not control for the number of individuals or the sex ratio in each tank in the mixed experiments, as we expected the sex ratio to be similar to that found in natural conditions. All containers were exposed to continuous light for the first 3 days after hydration, followed by a light regime of 10 h light and 14 h dark day-night rhythm. Containers were regularly topped up to compensate for evaporation. Individuals were fed daily with fish food and reared until maturity at 20 °C (c. 4 weeks). After this period, individuals were collected and sexed (see above) and containers left to dry. Eggs laid in the isolation experiment containers were collected and counted under a stereomicroscope. Adult individuals were snap-frozen and then thawed to isolate the eggs they were carrying. Immediately after collecting the eggs, the individuals were preserved in ethanol and the eggs were allowed to dry under room conditions and stored in plastic tubes for subsequent genetic analysis (see below).

#### *Segregation analyses*

We tested the segregation patterns of genetic markers of the offspring produced by isolated individuals and by individuals with mixed rearing, where males were not found against the expected genotypic frequencies under hermaphroditism and various modes of parthenogenesis (Pearcy, Hardy & Aron, 2006). We used locus tcAC-8p1 developed by Cesari *et al.* (2004) for segregation analysis as this microsatellite was polymorphic in populations used in the reproduction experiments. We performed segregation analyses in reproducing individuals as follows. First, we carried out a pre-screening of locus tcAC-8p1 in individuals that produced eggs (see above for DNA extraction and microsatellite amplification and typing procedures). After this, 20 eggs produced by individuals found to be heterozygote were typed for the microsatellite locus. We examined the genotypes of eggs

typed for each heterozygote individual and tested their genotype proportions against expectations of hermaphroditism or parthenogenesis using chi-square tests (see Pearcy *et al.*, 2006).

## **Results**

### *Proportion of males in populations*

The only sample from a gonochoric population (ESP) showed a proportion of 53% of males. Among the other samples the proportion of males ranged from 0% in NEU, GOD and KOE21 up to a maximum of 10% in KOE27 (Table 2).

### *Population genetic diversity*

We screened a total of 324 individuals from 10 populations (see Table 2). The number of alleles per microsatellite loci varied between three and 10 across all samples. All populations showed low levels of genetic diversity (Tables 2, Appendix S1). Surprisingly, the gonochoric Espolla population did not show higher genetic variability than populations with low proportion of males when comparisons were made using five microsatellite loci. Permutation tests further revealed that neither heterozygosity and allelic richness nor  $F_{IS}$  was significantly different between populations with a low proportion or no males ( $P > 0.3$ ).

### *Population structure*

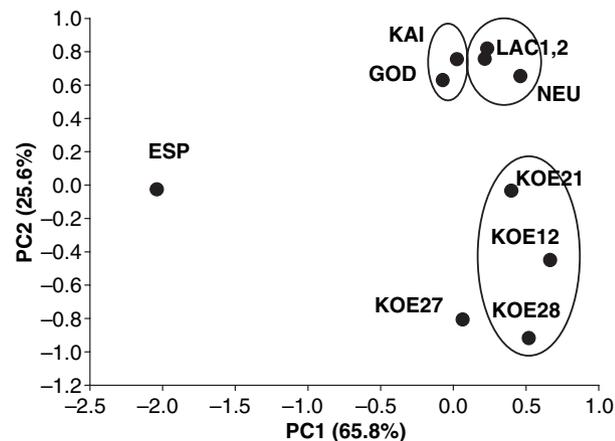
All populations with at least one polymorphic locus, except for LAC1, showed significant deviations from Hardy–Weinberg equilibrium due to heterozygote deficiencies. The lowest coefficient of inbreeding ( $F_{IS}$ ) was found in Espolla (0.287). In populations without males,  $F_{IS}$  was higher and ranged from 0.456 (KOE12) to 1.000 (KAI). LAC1 did not deviate from Hardy–Weinberg proportions, but was also one of the populations with smallest sample size (13 individuals typed) and had a single polymorphic locus. Global multilocus  $F_{ST}$  was 0.82 ( $P < 0.0001$ ) and most pairwise  $F_{ST}$  values were significant (see Table 3), even between populations occupying nearby ponds (such as the four from the Koenigswartha area, KOE). LAC1 and LAC2, on the one hand, and NEU and LAC2 did not show significant  $F_{ST}$ . The first two axes of a  $F_{ST}$

**Table 3** Population differentiation in *Triops cancriformis*. Pairwise between-population  $F_{ST}$  is shown in the lower hemimatrix. The upper hemimatrix shows the significance of tests for pairwise population differentiation not assuming Hardy–Weinberg equilibrium within populations

	ESP	KOE12	KOE27	NEU	GOD	KAI	LAC2	KOE21	LAC1	KOE28
ESP	–	**	**	**	**	**	**	**	**	**
KOE12	0.7193	–	**	**	**	**	**	*	**	**
KOE27	0.5144	0.2017	–	**	**	**	**	**	**	**
NEU	0.7568	0.4420	0.4068	–	**	**	NS	**	**	**
GOD	0.6935	0.5148	0.3497	0.3229	–	*	**	**	**	**
KAI	0.7461	0.5395	0.3846	0.2402	0.0216	–	*	**	**	**
LAC2	0.8335	0.5404	0.4274	0.0347	0.3163	0.2395	–	**	NS	**
KOE21	0.7562	0.0977	0.2116	0.4218	0.3763	0.3981	0.4490	–	**	**
LAC1	0.8047	0.4992	0.3944	0.0115	0.2544	0.1975	0.0937	0.3679	–	**
KOE28	0.8455	0.1323	0.2195	0.8351	0.7314	0.7971	0.9394	0.3696	0.8711	–

NS, not significant; \*\*Highly significant,  $P < 0.001$ ; \*Significant,  $P < 0.05$ .

based PCA cumulatively explained 91% the total variation (Fig. 1). The differentiation between ESP and other populations accounted for most of the variation on PC1, showing that this was overall genetically the most distinct population. Most of the variation on PC2 was due to the differentiation among four samples from Koenigswartha and between these and the remaining samples. The remaining Central European populations formed a relatively closely related cluster. These results were partly reflected in the Bayesian cluster analysis, which found that the most probable



**Fig. 1** Principal component analysis based on pairwise  $F_{ST}$  values (global  $F_{ST} = 0.67$ ) calculated over the five common loci in the 10 *Triops cancriformis* populations analysed. Results of the Bayesian cluster analysis are also indicated. Populations within each circle were identified as belonging to the same cluster. Populations not in circles represented single-population ‘clusters’.

number of genetic units ( $K$ ) was 5 ( $P = 0.96$ ). Populations ESP and KOE27 formed individual clusters, whereas the remaining three Koenigswartha populations formed a separate cluster. The Bayesian analysis identified two independent clusters in the remaining populations, one containing a group of populations which appeared to be closely related based on the  $F_{ST}$  analysis (LAC1, LAC2 and NEU), and the other an English population (GOD) and a German population (KAI).

Mantel tests and reduced major axis regression analyses showed a significant, but weak, negative correlation between genetic and geographic distance ( $P = 0.025$ ,  $R^2 = 0.16$ ) when the Espolla population was included. The test was non-significant when this most distant, most divergent population was excluded, indicating that the pattern was likely to be driven by the Espolla population.

#### Linkage disequilibrium

Due to the limited polymorphism within most populations, linkage disequilibrium analysis was only carried out for the four populations screened for eight loci (ESP, KOE12, KOE27 and NEU). For the gonochoric ESP population, just one out of 18 (5.5%) comparisons was significant ( $P = 0.044$  between tcCA-6p5 and tcGA-8p8). For the two populations with a low proportion of males, KOE12 showed linkage disequilibrium for four out of 10 comparisons (40%) and KOE27 showed highly significant linkage for seven out of 21 comparisons (33.3%). Surprisingly,

for the NEU population, where no males were found, linkage was reduced with just two significant disequilibria found out of 15 (6.6%).

No sex-linkage was found for any locus (data not shown). Even in locus tcAC-8p1 [which was found by Mantovani *et al.* (2008) to be sex linked in their samples from Espolla] the common genotype was present in both males and females and the rare genotype was found in one female.

#### Reproduction experiments

Isolated individuals from the non-gonochoric populations tested (KOE12, KOE27 and NEU) produced eggs (Table 4). Isolated Espolla females either failed to produce eggs or produced what appeared to be unfertilised eggs that did not develop properly, as they were only found at the front of the brood pouch remained yellow and lacked the reddish eggshell. In contrast, all individuals in mixed cultures produced eggs, including the Espolla population, and they seemed developed normally. Given that the conditions were not comparable between the isolation and mixed experiments, no statistical comparison of the numbers of eggs produced was carried out, although

more eggs were always produced in the mixed experiments.

#### Segregation analyses

We established the reproductive mode in a family based genetic analysis, which included the genotyping of eggs from individuals hatched from sediment sampled from natural populations and reared in isolation or in the absence of males in the laboratory. Following genotyping at locus tcAC-8p1 we selected 12 individuals, (eight from KOE12, with a low proportion of males and two from KOE21, no males) which were heterozygous and carried a sufficient number of eggs to screen the offspring (Table 5). In all cases, homozygotes and heterozygote eggs were retrieved ( $n = 200$  eggs screened), and the percentage of homozygotes in the offspring was close to 0.50 (0.46, 0.48). We tested for deviations from expectations of segregation of locus tcAC-8p1 in the offspring resulting from different reproductive modes (Table 6). Results deviated significantly from expectations of apomictic reproduction (true asexual parthenogenesis) and three modes of automixis (meiotic parthenogenesis), gamete duplication, central fusion and

**Table 4** Results of isolation/mixed rearing experiments in *Triops cancriformis* populations. Several mixed rearing experiments were completed for KOE12, NEU and GOD and the results are presented in different lines

Population	Experiment							
	Isolation				Mixed			
	$N_{fm}$	$N_m$	Prop. of males	$N_e$ (SD)	$N_{fm}$	$N_m$	Prop. of males	$N_e$ (SD)
ESP	14	21	0.60	*	29	41	0.59	6.78 (7.10)
KOE27	6	2	0.25	18.33 (7.66)	55	1	0.02	19.55 (8.00)
KOE12	10	1	0.09	29.43 (13.64)	3	5	0.63	44.60 (42.37)
					5	0	0	
					1	0	0	
					5	0	0	
					4	2	0.33	
					2	0	0	
KAI	/	/			15	1	0.06	41.86 (13.23)
NEU	53	0	0	22.72 (16.25)	5	0	0	46.67 (29.14)
					44	0	0	13.00 (10.31)
GOD	/	/			19	0	0	52.00 (24.76)
					6	0	0	21.67 (9.44)
KOE21	/	/			15	0	0	51.73 (30.85)

$N_{fm}$ , number of females reared;  $N_m$ , number of males reared;  $N_e$ , average number of eggs produced per female/hermaphrodite; SD, standard deviation.

\*Eggs in front of brood pouch, remain yellow, not cemented with reddish eggshell.

**Table 5** Results of the TcAC-8p1 microsatellite typing for eggs of individual *Triops cancriformis* bearing eggs from two non-gonochoric populations (KOE12 and KOE21)

Population	KOE12										KOE21				
	1*	8a*	11*	18*	3	7	8b	94	10	92	Total	1*	10	Total	
Individual N	17	16	12	14	15	16	11	7	5	11	124	17	16	33	
Genotype															
147/147	0	3	1	1	7	4	2	2	3	1	24	4	3	7	
147/151	15	11	10	7	4	6	3	3	1	7	67	9	8	17	
151/151	2	2	1	6	4	6	6	2	1	3	33	4	5	9	

Individuals labelled with an asterisk were grown in mixed rearing experiments where males were absent.

**Table 6** Segregation analyses for locus TcAC-8p1 in *Triops cancriformis* reproduction experiments and consistency with different non-gonochoric reproductive modes. The observed number of genotypes in the offspring of individuals from two populations is given. The  $r$  index, or expected homozygosity in the offspring of a heterozygote mother under different reproductive modes from selfing to automixis and apomixis, is given and, below this, the significance of a chi-square test of consistency of observed versus expected genotypes

Population	N	Observed genotypes in offspring			Selfing ( $r = 0.5$ )	Automixis				Apomixis ( $r = 0$ )
		147/147	147/151	151/151		Gamete duplication ( $r = 1$ )	Terminal fusion ( $r = 1/3-1$ )	Central fusion ( $r = 0-1/3$ )	Random fusion ( $r = 1/3$ )	
KOE12	124	24	67	33	NS	**	NS	**	**	**
KOE21	33	7	17	9	NS	**	NS	*	*	**
Total	157	31	84	42	NS	**	NS	**	**	**

NS, not significant; \*\*Highly significant,  $P < 0.001$ ; \*Significant,  $P < 0.05$ .

random fusion. The results were compatible with selfing hermaphroditism for both populations, but also with a mode of automictic parthenogenesis (terminal fusion) in which the products of the second meiotic division fuse to reconstitute diploidy. In this mode of meiotic parthenogenesis, the frequency of homozygotes ( $r$ ) in the offspring of isolated individuals varies from 1/3 to 1, depending on the distance of each particular locus to the centromere, which determines the likelihood of recombination: the closer a locus is to the centromere the more likely it is that the offspring will be homozygous (Pearcy *et al.*, 2006).

## Discussion

### Genetic population structure

The results showed that European *Triops cancriformis* populations are characterised by strong Hardy-Weinberg disequilibria and reduced genetic diversity regardless of their reproductive mode, although this

pattern is more pronounced in non-gonochoric populations. This matches expectations under selfing hermaphroditic reproduction but also suggests that processes other than inbreeding due to selfing have contributed to the observed low levels of genetic diversity. The extremely high levels of population differentiation, and weak or absent patterns of isolation by distance, indicate that contemporary gene flow is much reduced and drift is strong, maybe due to strong founder effects (Hutchison & Templeton, 1999). In contrast to such differentiation in microsatellite loci, a previous study showed that the investigated populations are closely related according to their mtDNA (Zierold *et al.*, 2007) and are likely to have recolonised Northern and Central Europe after the last glacial maximum. Together, mtDNA and microsatellite results suggest that this postglacial colonisation was associated with strong bottlenecks, causing divergence between source and sink populations on a nuclear genetic level. Low levels of gene flow after colonisation would also have been required to

maintain such a high level of differentiation. Such a pattern has been found even in aquatic species with high dispersal capacity – and high inferred levels of actual dispersal – such as rotifers, cladocerans and *Artemia* (Gómez & Carvalho, 2000; De Meester *et al.*, 2002; Muñoz *et al.*, 2008) and has been explained by the Monopolisation Hypothesis (De Meester *et al.*, 2002) as attributable to persistent founder effects due to rapid build-up of large population sizes and local adaptation. Evidence for dispersal in *Triops* is lacking and, despite their resistant diapausing eggs, it might not be expected to be high, due to the fact that their habitats are often of reduced size and have a temporary and unpredictable character and their eggs are larger than those of rotifers and cladocerans. If interpopulation dispersal is not important, then genetic founder effects and additional genetic drift due to population size fluctuations would suffice to explain the strong population structure we found in *T. cancriformis*, and this could also be the case in some anostracans (Ketmaier *et al.*, 2005, 2008). In the case of *Triops*, selfing hermaphroditism could have further contributed to increase genetic drift. Interestingly, Koenigswartha populations, which in our previous phylogeographic survey (Zierold *et al.*, 2007) showed the co-occurrence of two mtDNA lineages in varying proportions, showed the highest level of differentiation in the non-gonochoric populations.

#### Reproductive mode

The extremely low genetic variation within *Triops* populations made the inference of its reproductive mode from genetic data very challenging. First, the proportion of suitable (heterozygote) females that could be screened in our marker inheritance experiments was very limited. Furthermore, only one locus could be used and, therefore, a distinction between selfing and automixis could not be made. Second, the low overall genetic diversity in natural populations reduced the power to detect signatures of outbreeding in relation to the proportion of males. This has also been recognised in other animals with high selfing rates, and can result in a publication bias of results for animal species with high selfing rates (Jarne & Auld, 2006).

Nevertheless, significant departures from Hardy–Weinberg expectations were found in most populations, with high inbreeding coefficients and strong

linkage disequilibria, patterns consistent with high levels of selfing in populations. The significant  $F_{IS}$  in the Espolla population was unexpected because individuals are unable to reproduce in isolation (this paper and also reported in Korn *et al.*, 2006). Such a pattern could be an indication of inbreeding due to low effective population size or of a Wahlund effect due to hatching of individuals produced in different breeding seasons from cyst banks (Gómez & Carvalho, 2000; De Meester *et al.*, 2002). *Triops* shows a bet-hedging hatching strategy in which not all eggs hatch when the conditions become favourable (Takahashi, 1977; Simovich & Hathaway, 1997) and diapausing cysts are long-lived (Lauterborn, 1921; Thiery, 1997) conditions which favour the accumulation of cyst banks. A recent survey of genetic variation in this population by Mantovani *et al.* (2008), however, found that samples of two independently analysed year cohorts of this population were in HW equilibrium.

Although the gonochoric population showed the highest genetic diversity and lowest level of inbreeding, we did not find a significant correlation between genetic diversity and male proportion when the gonochoric population was excluded. In contrast Sassaman *et al.* (1997) showed a clear relationship between reproductive mode and genetic diversity at allozyme loci in *Triops longicaudatus* (LeConte) and *T. newberryi* (Packard). The discrepancy between our data and theoretical expectations could have been due to: (i) the low number of loci and populations surveyed in this study, (ii) the extremely reduced diversity of European populations, and (iii) inaccurate estimation of the proportion of males due to sampling error or temporal fluctuations. We screened from 19 to 102 individuals in those populations where we found no males, but records exist of *Triops* populations with few males such that the latter could easily be missed in small samples (Sassaman *et al.*, 1997; Zierold *et al.*, 2007).

The segregation analysis of 200 eggs produced by 12 individuals from two populations indicated that hermaphroditism, and not apomixis, is the most likely reproductive mode of European *Triops* populations. Our results cannot rule out that the individuals investigated reproduce through some form of automictic parthenogenesis (Percy *et al.*, 2006). Such a mechanism appears unlikely, however, given that no mechanism of diploidy restoration has been described hitherto in *Triops* (Sassaman, 1991). More loci would

need to be investigated in order to rule out this possibility. However, our segregation analysis showed that the females from populations with no males or a low frequency of males did not reproduce asexually (through ameiotic parthenogenesis) since the presence of homozygote offspring in substantial frequencies is incompatible with such a hypothesis. In summary, both population genetic data and results from the segregation analysis are consistent with androdioecious/selfing hermaphroditic populations throughout most of the range of *T. cancriformis*. The presence of testicular lobes in individuals from most of the populations without males (Longhurst, 1955b,c; Zaffagnini & Trentini, 1980), and evidence of meiosis (Scanabissi & Trentini, 1979), further indicates that non-gonochoric *T. cancriformis* are made of self-fertilising hermaphrodites, like the American species *T. newberryi* (Sassaman *et al.*, 1997), and not automictic parthenogens. Other large branchiopod species previously considered automictic parthenogens, such as the Spinicaudatan *Limnadia lenticularis* (Linnaeus) (Zaffagnini, 1969) and the best studied androdioecious branchiopod *Eulimnadia texana* (Packard) (see Weeks & Zucker, 1999) have eventually been shown to be composed of self-fertilising hermaphrodites with no or few males present in populations, which is indicative of androdioecy (Eder *et al.*, 2000; Scanabissi & Mondini, 2002). A study of much larger scope would be needed to assess if there are developmental changes in the reproductive mode of individuals, or there is some form of environmental determination, or if the mechanism resembles that described in *Eulimnadia* or *Triops newberryi*. Further, it is unknown if there are mixtures of different reproductive strategies in populations (i.e. pure females with males and hermaphrodites).

#### Sex-linkage

Hermaphrodites in the androdioecious congeneric species *T. newberryi* and in *E. texana* are heterogametic, that is, sex-linked markers and gender inheritance indicates that hermaphrodites can be of two genetic types (one which breeds true and the other that produces males and hermaphrodites in a 1 : 3 ratio) (Sassaman, 1991, 1995). This suggests that hermaphrodites have a chromosome or chromosomal region/locus of the type WZ or WW, whereas males are homogametic (ZZ). In contrast to this expectation,

Mantovani *et al.* (2008) indicated that the Espolla females they scored for locus tcAC-8p1 were homozygotes for the most common allele, whereas males had any genotype, suggesting sex linkage of this locus but female homogametic status. Our findings for the same locus are not compatible with this interpretation, however, as Espolla males and females did not differ in their genotypic composition. None of our loci showed evidence of sex-linkage.

In conclusion, our dataset showed reduced genetic diversity in northern European *T. cancriformis* populations and a strong level of inbreeding and linkage disequilibrium. Populations were highly differentiated, probably due to strong founder effects. The combined interpretation of population genetic data and segregation analysis in our study indicated that reproducing individuals from populations in Northern and Central Europe, which contain no or few males, are probably hermaphrodites.

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### Supporting Information

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

**Appendix S1.** Population genetic diversity in *Triops cancrivormis* populations. Sample size per locus, number of alleles, observed and expected heterozygosity and fixation index per locus are given.

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