A peer-reviewed version of this preprint was published in PeerJ on 17 June 2014.

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Maccari M, Amat F, Hontoria F, Gómez A. 2014. Laboratory generation of new parthenogenetic lineages supports contagious parthenogenesis in *Artemia*. PeerJ 2:e439 https://doi.org/10.7717/peerj.439

Laboratory generation of new parthenogenetic *Artemia* lineages through contagious parthenogenesis

Contagious parthenogenesis – a process involving rare functional males produced by a parthenogenetic lineage which mate with coexisting sexual females resulting in fertile parthenogenetic offspring – is one of the most striking mechanisms responsible for the generation of new parthenogenetic lineages. Populations of the parthenogenetic diploid brine shrimp *Artemia* produce fully functional males in low proportions. The evolutionary role of these so-called *Artemia* rare males is, however, unknown. Here we investigate whether new parthenogenetic clones could be obtained in the laboratory through contagious origin. We assessed the survival and sex ratio of the hybrid ovoviviparous offspring from previous crosses between rare males and females from all Asiatic sexual species, carried out cross-mating experiments between F1 hybrid individuals to assess their fertility, and estimated the viability and the reproductive mode of the resulting F2 offspring. Molecular analysis confirmed the parentage of hybrid parthenogenetic F2. Our study documents the first generation of parthenogenetic lineages through contagious parthenogenesis in *Artemia*. We discuss the possible genetic mechanisms responsible for parthenogenesis and the likelihood of contagious parthenogenesis in natural environments.

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.333v1 | CC-BY 4.0 Open Access | received: 31 Mar 2014, published: 31 Mar 2014

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Introduction

Parthenogenesis in animals has evolved through different molecular mechanisms that influence the initial genetic variability of parthenogenetic strains and therefore have important implications on their evolutionary success and persistence (Simon et al., 2003). One of the most striking mechanisms responsible for the generation of new parthenogenetic lineages is contagious parthenogenesis (Simon et al., 2003; Shön et al., 2009). This involves a parthenogenetic lineage able to produce functional males, which mate with coexisting sexual females producing fertile parthenogenetic hybrid offspring. The new parthenogenetic lineages will capture genetic diversity from the maternal sexual species, but will share some common genomic background from their parthenogenetic ancestor, linked to the parthenogenesis-inducing alleles (Simon et al., 2003; Tucker et al., 2013). This mechanism has been documented in aphids and parasitoid wasps (Schneider et al., 2002; Sandrock & Vorburger, 2011; Delmotte et al., 2013), and most extensively in the *Daphnia pulex* species complex (Innes & Hebert, 1988; Paland et al., 2005). In North American *D. pulex* parthenogenetic lineages, at least two distinct unrecombined haplotypes in chromosomes VIII and IX are implied in the sex-limited meiosis suppression (Lynch et al., 2008; Eads et al., 2012; Tucker et al., 2013). These haplotypes leading to obligate parthenogenesis in *D. pulex* stem from a single recent event of hybridization from its sister taxon *D. pulicaria* (Xu et al., 2013; Tucker et al., 2013). Multiple new parthenogenetic lineages have arisen since this event as males produced by asexual lineages spread these parthenogenesis-inducing haplotypes by mating with sexual females.

Artemia, an anostracan branchiopod commonly known as brine shrimp, is a typical inhabitant of hypersaline inland lakes and costal lagoons and salterns. This genus includes sexual species and lineages of obligate parthenogenetic populations of diverse ploidy

(Abatzopoulos, 2002), which makes it a good model system to investigate evolutionary transitions between reproductive systems. Parthenogenetic populations are restricted to the Old World where co-occur with several sexual species (Abatzopoulos, 2002; Agh et al., 2007; Abatzopoulos et al., 2009; Maccari et al., 2013a). All strains of *Artemia* can reproduce either ovoviviparously, with the release of free-swimming nauplii, or oviparously with the production of diapause cysts (Browne, 1980; Abatzopoulos, 2002).

Parthenogenetic diploid *Artemia* populations reproduce through automictic parthenogenesis (Abreu-Grobois, 1987), but produce fully functional males in low proportions (Stefani, 1964; Bowen et al., 1978; MacDonald & Browne, 1987; Maccari et al., 2013a). Crucially, these 'rare males' generate viable offspring when crossed with females of sexual Asiatic species (Bowen et al., 1978; Cai, 1993; Maccari et al., 2013a), to which they are closely related genetically (Muñoz et al., 2010; Maniatsi et al., 2011; Maccari et al., 2013b). However, the evolutionary role of rare males in the generation of *Artemia* parthenogenetic lineages is still unknown (Maccari et al., 2013a). Contagious parthenogenesis has been suggested in light of the genetic diversity of diploid parthenogenetic lineages (Maccari et al., 2013b), but we do not know if rare males are able to transmit parthenogenesis to their offspring, which is a requisite for contagious parthenogenesis to occur. In an early study, Bowen et al. (Bowen et al., 1978) crossed two parthenogenetic rare males, one from Yamaguchi (Japan) and the other one from Madras (India), with one sexual female of *A. urmiana* and one *A. franciscana* respectively, but they concluded that the genes for parthenogenetic reproduction could not be transmitted through males because they failed to obtain parthenogenetic hybrid F1 or F2 (and a F2 backcross).

Laboratory generation and establishment of unisexual lineages can be a useful tool to complement phylogenetic approaches to identify the mechanism involved in the transition from sexual to parthenogenetic reproduction. However, often most laboratory hybrids exhibit

low fertility and survival, or show deformation and abnormalities (Vrijenhoek, 1989; Mantovani et al., 1996). In vertebrates, the first successful laboratory generation of a unisexual hybrid involved the origin of the hybridogenetic fish *Poeciliopsis monacha-lucida* through crosses of *P. monacha* females with *P. lucida* males (Schultz, 1973). Laboratory hybrids of hemiclonal European water frog *R. esculenta* (*Rana ridibunda* x *Rana lessonae*) show even faster larval growth, earlier metamorphosis, and higher resistance to hypoxic conditions compared with their parental species and the equivalent hybrids in nature (Hotz et al., 1999). More recently, Lutes et al. (2011) generated self-sustaining tetraploid lineages of parthenogenetic lizards by pairing males of diploid sexual species *Aspidoscelis inornata* with females of the triploid parthenogenetic species *Aspidocelis exsanguis*. In invertebrates, the first laboratory generation of clonal hybrids in *D. pulex* was obtained by mating males from obligately parthenogenetic clones with cyclically parthenogenetic females (Innes & Hebert, 1988). In addition, new lineages of thelytokous parthenogenetic lineages have been obtained in the wasp *Lysiphlebus fabarum* and in a South African honeybee*, Apis mellifera capensis* (Lattorff et al., 2005; Sandrock & Vorburger, 2011).

Here we assess the reproductive role of *Artemia* rare males investigating whether new parthenogenetic clones could arise in laboratory through contagious origin. For this purpose, (1) we assess the survival and sex ratio of the hybrid ovoviviparous offspring from the crosses from (Maccari et al., 2013a) between rare males and Asiatic sexual species, (2) we carry out cross-mating experiments between these F1 hybrid individuals to assess their fertility, (3) we estimate the viability and the reproductive mode of the resulting F2 offspring; (4) finally we demonstrate genetically that parthenogenetic F2 are indeed the descendants of the original crosses. This study shows that *Artemia* has the potential of generating parthenogenetic strains through contagious parthenogenesis.

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Materials and methods

Populations and mating experiments

In a previous study, we set up mating experiments between rare males from the diploid parthenogenetic *Artemia* population from Bagdad (Iraq, hereafter PD) and sexual females from Central Asian *Artemia* species to assess the fertility and the reproductive potential of rare males (Maccari et al., 2013a). The females used were from the sexual Asiatic populations, *A. urmiana* from Koyashskoe Lake (Ukraine, URM), *A. sinica* from Yuncheng Lake (China, SIN), *A. tibetiana* from Lagkor Co Lake (Tibet, TIB) and *Artemia* sp. from Kazakhstan (*Artemia* Reference Center code – ARC1039, unknown locality, KAZ). These interspecific crosses resulted in viable ovoviviparous and oviparous F1 offspring with similar or higher viability than controls (intraspecific sexual crosses)(Maccari et al., 2013a).

Survival rate, sex ratio and reproductive performance of hybrid generations

For this study, live nauplii obtained from each ovoviviparous F1 hybrid brood were reared separately in jars containing brine at 80 gL^{-1} salinity, kept at 20–24 ºC under mild aeration at a 12D:12L photoperiod and fed a mixture of *Dunaliella* sp and *Tetraselmis* sp. (1:1) microalgae every other day. When animals showed signs of reproductive maturity they were counted and sexed to estimate survival rates (the proportion of individuals in a brood attaining adulthood) and sex ratio (the proportion of males in a brood). For this procedure the animals were placed in Petri dishes with seawater and anaesthetized with a few drops of freshwater saturated with chloroform and examined carefully under a binocular microscope. We tested for deviations from a 50% sex ratio per cross and per brood using a Chi-square goodness of fit test (Pearson's statistic)(Wilson & Hardy, 2002).

Reproductive performance of the F1 hybrid individuals was evaluated in F1xF1 cross fertility tests. For this purpose, 24 randomly size-matched hybrid F1 male-female pairs from

each cross were transferred into separate small glass beakers (60 ml) under the culture conditions described above. Quantitative and qualitative reproductive outputs of each pair were monitored every other day during culture medium renewal events. For each paired F1 female we counted the number of unfertilized and fertilized broods, distinguishing the latter in oviparous and ovoviviparous broods. In ovoviviparous offspring we also recorded the number of live and dead nauplii, and the number of abortive embryos (pale yellow-orange eggs). When oviparous offspring was produced, we counted the number of normally shelled diapausing cysts (pale grainy surface floating in 200 gl^{-1} brine), as opposed to abortive, abnormally shelled embryos (bright brown colour cysts sinking in 200 gl^{-1} brine)(Maccari et al., 2013a).

Emerged F2 hybrid nauplii were reared until maturity as described above. They were counted and sexed to estimate their survival rate and sex ratio in the F2 generation. Then, males and females were individually isolated in containers to check if females could reproduce in isolation, as would be expected in parthenogenetic individuals.

Paternity analysis of parthenogenetic F2 individuals

a) Microsatellite analysis

The F2 hybrid generation resulting from crosses between rare males and sexual females from *A. urmiana* and *Artemia* sp. from Kazakhstan included parthenogenetic individuals. In order to rule out contamination and confirm that they were F2 individuals resulting from the original crosses, we screened three microsatellite loci, previously screened in the parental individuals in another study (Maccari et al., 2013a), in the parthenogenetic F2 animals obtained. Each microsatellite locus (Apdq02TAIL, Apdq03TAIL and Apd05TAIL) (Muñoz et al., 2008) was amplified separately in PCRs performed as described in Maccari et al. (2013a). Alleles were scored using the CEQ Fragment Analysis software (Beckman Coulter™) and

checked manually. If F2 individuals had a paternal allele in any of the loci this would confirm that they were descendants of the diploid parthenogenetic rare males.

b) Maternal lineage

The F2 resulting from the rare male x sexual female cross and F1 x F1 cross should carry the maternal DNA of the sexual strain. To establish the maternal lineage of the parthenogenetic F2 offspring, a 709-bp fragment of mitochondrial cytochrome *c* oxidase subunit I (COI) gene region was amplified in the parental individuals, in the F1 offspring and in the parthenogenetic animal obtained in the second generation. Total DNA was extracted and PCR was carried out as described previously (Maccari et al., 2013a). PCR amplifications were sent to MACROGEN for sequencing, and the resulting electrophoregrams were checked by eye using CodonCode Aligner v. 3.5 (CodonCode Corporation, Dedham, MA).

Results

Survival rate and sex ratio of F1 hybrid offspring

A total of 102 ovoviviparous hybrid F1 broods produced by the crosses between each combination of sexual species with rare males (Maccari et al., 2013a) were reared to maturity. The live nauplii obtained in each brood were morphologically normal. Survival rates to adulthood were over 50% in all F1 hybrid offspring (Figure 1), and were highest in the F1 PD x SIN (80%), and lowest in F1 PD x URM and F1 PD x TIB (ca. 56%)(for the codes of the hybrid crosses see Figure 1). The F1 offspring sex ratio ranged from 49% males in F1 PD x KAZ cross to 53% males in F1 PD x TIB cross and did not significantly differ from 50% in any cross.

Reproductive performance of F1 hybrid offspring

Prior to setting up the crosses, all females were isolated from males for two weeks to ensure that they could not reproduce in isolation (i.e. they were sexual females). No F1 females were able to reproduce when isolated from males. Then, a total of 24 mating pairs (F1 hybrid female x F1 hybrid male) were set up for each F1 produced in each combination of sexual species with rare males. As some individuals died before mating, the final number of experimental pairs ranged from 10 to 22 per cross, which produced a total of 173 fertile and 92 infertile F2 hybrid broods (Table 1). Ovoviviparous and oviparous F2 offspring viability is shown in Figure 2. The percentage of abortive embryos was high in all crosses (between 70% and 90%), while the proportion of live nauplii in all hybrid ovoviviparous broods was low (from 5% to 25%). In oviparous broods, the proportion of properly shelled cysts ranged from 25% in F2 PD x TIB to 61% in F2 PD x URM.

Survival rate and sex ratio of F2 hybrid offspring

A total of 103 F2 ovoviviparous broods were recorded (Table 1), of which 35 broods, characterized by the greatest number of nauplii, were monitored to assess the survival rate and the sex ratio of the F2 offspring. F2 nauplii were morphologically normal but they had low survival rates when compared to F1 nauplii (Figure 3). No F2 hybrid offspring produced by the crosses between rare male and *A. tibetiana* survived to maturity. The F2 PD x KAZ had the highest survival rate, about 37%, followed by the F2 PD x SIN (34%) and F2 PD x URM (24%). The overall sex ratio per cross was significantly female-biased in F2 PD x KAZ and F2 PD x URM crosses (respectively 12% and 22%), but was non-significantly different from 50% in the F2 PD x SIN (43%). Furthermore, we observed differences in the sex ratio of the F2 offspring among different pairs from the same cross, in particular for F2 PD x KAZ and F2 PD x URM crosses (see Table 2). In the cross F2 PD x KAZ, which higher brood sample sizes, one brood had an even sex ratio and the remaining six were female biased.

Generation of hybrid parthenogenetic individuals

Some females isolated from males of all 35 F2 hybrid broods (when males were present) reproduced parthenogenetically in two of the three hybrid lineages. Specifically, 12 out of 41 isolated females were parthenogenetic in F2 PD x KAZ (in four of five broods), and two out of 36 isolated females in F2 PD x URM (in two of five broods). None of the 21 F2 PD x SIN broods included females that could reproduce in isolation.

Paternity analysis

In order to examine the parentage of newly generated hybrid parthenogenetic individuals we integrated the information from the mitochondrial COI and from microsatellites markers, (Table 3). Six of the 10 analysed females from brood 4 of the cross F2 PD x KAZ were parthenogenetic and produced F3 clones. As expected, all of them shared their mtDNA haplotype with their sexual grandmother, and amplified one paternal allele in the two informative microsatellite loci, confirming that they were the offspring of the rare male used in the crosses. The F3 generation was overall composed by females and by two rare males with the same genotype than their F2 mothers.

Each of the two analysed F2 broods from the crosses PD x URM (broods 4 and 7), composed by three and 13 females respectively, included a parthenogenetic female that produced F3 parthenogenetic clones. In both cases, the F2 parthenogenetic female shared its COI haplotype with its sexual grandmother. In one cross, one paternal allele was detected in the F2 hybrid female at each of the three microsatellite loci; in the other cross, the parthenogenetic female inherited one paternal allele at the two informative loci. Most individuals of the F3 generation, composed by females and one rare male in both crosses, have the same genotype of their F2 mothers, with a few exceptions that lacked one of the maternal alleles.

Discussion

This study reports for the first time the laboratory generation of parthenogenetic *Artemia* lineages through hybridization via rare males, i.e. through contagious parthenogenesis (Simon et al., 2003), shedding light into the possible evolutionary role of parthenogenetically produced males in the genus.

Contagious parthenogenesis has important evolutionary consequences as it results in the repeated generation of new asexual genotypes, increasing the genetic diversity in parthenogens. This counteracts the loss of asexual genotypes resulting from the accumulation of deleterious mutations (Muller's ratchet) or gene conversion (Tucker et al., 2013) and could contribute to the evolutionary success of parthenogenesis (Simon et al., 2003).

The occurrence of contagious parthenogenesis relies on regular or occasional hybridization or absence of complete reproductive isolation between parthenogenetically produced males and closely related sexual females (Simon et al., 2003). In a previous study, we showed the absence of prezygotic isolation between rare males and Asiatic sexual *Artemia* species since these males often coexist in the same environment of a sexual species, show normal pairing behaviour and are fully functional and capable of fertilizing eggs from females of sexual Asiatic *Artemia* species producing viable hybrid offspring (Maccari et al., 2013a). Under laboratory conditions, each combination of sexual species with rare males produced morphologically normal, viable hybrid F1 sexual generations. Their survival rate to adulthood was over 50% for all the hybrid populations, a good value of survivorship for all *Artemia* species (Browne & Wanigasekera, 2000).

Females constitute approximately the 50% of each F1 hybrid population, an even sex ratio that usually characterizes *Artemia* sexual populations, and this was confirmed by their inability to reproduce without males. Although all laboratory F1 lines were found to combine

ovoviviparous and oviparous reproduction, we observed a strong decline in the reproductive output in all crosses if we compared them with the reproductive performance of the parental crosses (Maccari et al., 2013a). Ovoviviparous broods were mostly made up by abortive embryos (more than 80%) in all the crosses and live nauplii represented only 25% of the offspring in the F2 PD x SIN hybrid generation, and less than 10% in all the other crosses (F2 PD x KAZ, F2 PD x URM and F2 PD x TIB). Oviparity, the production of dormant encysted embryos that are resistant to extreme environmental conditions, was represented by a variable quantity of properly shelled embryos, only 25% in the F2 PD x TIB increasing up to 61% in F2 PD x URM.

In contrast to the high survival rates of F1 hybrids, hybrid breakdown was evident in the F2 generation. Nauplii from F2 hybrid generations had low survival rates and were completely unviable in the F2 PD x TIB generation. The lower fertility level of F1 laboratory populations and the reduced viability of F2 hybrid individuals suggest partial genetic incompatibility between parthenogenetic males and sexual females. However, the production of some viable offspring both in F1 and F2 in all hybrid crosses is not so surprising given the recent evolutionary origin of diploid parthenogenetic lineages (Holocene) (Muñoz et al., 2010; Maccari et al., 2013b).

In two of three hybrid generations, F2 PD x KAZ and F2 PD x URM, we identified 14 hybrid females that upon reaching maturity were capable of parthenogenetic reproduction, as we would have expected considering the strongly female biased values of sex ratio for these two crosses. Genetic analysis confirmed the parentage of the parthenogenetic lineages found as the F2 individuals inherited the COI haplotype from the sexual grandmother but included some paternal alleles at nuclear markers, showing that they were the offspring of the rare male used in the crosses. Our results disagree with previous observations suggesting that rare

males in the genus *Artemia* are not capable to transmit parthenogenesis-inducing alleles (Bowen et al., 1978).

The production of parthenogenetic individuals only in the second hybrid generation, suggests that the parthenogenesis-inducing alleles are recessive in *Artemia*. A single-locus recessive inheritance of obligate parthenogenesis also occurs in *Apis mellifera capensis* and in *Lysiphlebus fabarum* (Engelstädter et al., 2011; Lattorff et al., 2005; Lattorff et al., 2007). If a single recessive locus was responsible for parthenogenesis and there was no differential viability in *Artemia*, we would expect a 25% of parthenogenetic individuals in the F2 generation. The proportion of isolated females that reproduced parthenogenetically differed between the crosses. In the cross F2 PD x KAZ, this proportion was 29%, whereas in the cross F2 PD x URM this was much lower (5.5%). These results suggest either differences in the mechanism underlying parthenogenesis between populations, or viability differences linked to the putative locus associated to parthenogenesis. The latter is suggested by the lower viability of F2 PD x URM nauplii. This is in contrast with *D. pulex*, where the sex-limited meiosis suppression genes are dominant and the asexual clones arise in the first hybrid generation (Innes & Hebert, 1988).

The ability of sexual females of *A. urmiana* and *Artemia* sp. from Kazakhstan to generate parthenogenetic clones when crossed with rare males is not surprising, as the two main mitochondrial haplogroups of diploid parthenogenetic *Artemia* lineages are related to these species (Muñoz et al., 2010; Maniatsi et al., 2011; Maccari et al., 2013b). Although repeated gene flow between sexual females and asexual males through contagious parthenogenesis would be expected to result in a regular emergence of asexual strains with diverse maternal origins, the fact that just two, possibly three, maternal origins of parthenogenetic lineages have been identified (Muñoz et al., 2010; Maniatsi et al., 2011; Maccari et al., 2013b) indicate that the incidence of contagious parthenogenesis must be extremely low in natural

environments. This could be due to the low percentage of male offspring of parthenogenetic females (Maccari et al., 2013a) or to the possibly lower fitness of newly emerging asexual strains comparing to sexual ones.

Our findings document the first generation of parthenogenetic lineages through contagious parthenogenesis in *Artemia*, providing evidence that contagious parthenogenesis can potentially occur in the genus *Artemia*. Demonstration of contagious parthenogenesis as the mechanism underlying parthenogenesis in *Artemia* in the wild will necessitate the use of genomic tools. Further studies on hybrid fitness would be necessary to estimate the strength of reproductive isolation and to compare the reproductive performance of contagious parthenogenetic clones with the parental parthenogenetic strains. The origin of independently reproducing parthenogenetic clones in laboratory raises the question either these clones could survive in nature with the sympatric sexual species.

Acknowledgements

We wish to thank Paul Nichols for his help with microsatellite screening and Mónica Barbosa, Eva Becerro and Diana Guinot for their help with the laboratory experiments. We thank Maria José Carmona for her constructive suggestions on a previous version of this manuscript. This study has been funded by the Plan Nacional CGL2008-project to FA, sponsored by Spanish Government MICIN. AG was supported by a National Environment Research Council (NERC) Advanced Fellowship (NE/B501298/1). MM was supported by a fellowship of the JAE Program from CSIC and European Social Fund.

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Table 1. Number of total, fertilized, ovoviviparous and oviparous broods in F1 hybrid offspring. F1 hybrids are from parental crosses between *Artemia urmiana* (URM), *Artemia sinica* (SIN), *Artemia tibetiana* (TIB), *Artemia* sp. from Kazakhstan (KAZ) and diploid parthenogenetic *Artemia* rare males (PD).

Cross	Pairs	Total broods	Fertilized broods	Ovoviviparous broods	Oviparous broods
F1 PD x KAZ	18	80	42	37	5
F1 PD x URM	16	48	26	22	4
F1 PD x TIB	10	33	18	4	14
F1 PD x SIN	22	104	87	40	47

Table 2. Sex ratio and parthenogenetic females found in 35 F2 PD x KAZ, F2 PD x URM and F2 PD x SIN *Artemia* broods. Asterisks ($P \leq 0.05$) indicate significant differences from 50% sex ratio (number of males/total individuals) (Chi-square goodness of fit test was employed). All females obtained were isolated to determine their mode of reproduction.

	Brood	Females	Males	Total	Sex ratio (%)	Parthenogenetic females
F2 PD x KAZ	*1*	10	0	10	0,00**	3
	2	10	2	12	16,67*	1
	3	7	8	15	53,33	0
	4	20	0	20	0,00**	6#
	5	4	0	4	0,00*	2
	6	64	2	66	3,03**	-
	7	31	1	32	3,13**	-
F2 PD x URM	*1*	16	3	19	15,79**	0
	2	2	4	6	66,67	0
	3	2	0	2	0,00	0
	4	3	1	4	25,00	1
	5	2	1	3	33,33	-
	6	2	0	2	0,00	-
	7	13	2	15	13,37**	1
F2 PD x SIN	*1*	4	4	8	50,00	0
	2	6	6	12	50,00	0
	3	5	3	8	37,50	0
	4	6	15	21	71,43*	0
	5	7	9	16	56,25	0
	6	6	3	9	33,33	0
	7	1	3	4	75,00	0
	8	1	1	2	50,00	0
	9	4	5	9	55,56	0
	10	9	6	15	60,00	0
	11	10	10	20	50,00	0
	12	5	6	11	54,55	0
	13	15	12	27	44,44	0
	14	23	24	47	51,06	0
	15	8	17	25	68,00	0
	16	22	24	46	52,17	0
	17	5	8	13	61,54	0
	18	16	0	16	0,00**	0
	19	7	0	7	0,00**	0
	20	4	1	5	20,00	0
	21	14	21	35	60,00	0

#Out of 10 isolated females.

Table 3. Mitochondrial cytochrome *c* oxidase subunit I (COI) and microsatellite loci analyses for F0 females and males (parental individuals), and for parthenogenetic F2 and F3 offspring obtained from the hybrid *Artemia* crosses. Genotypes for three microsatellite loci (allele sizes in base pairs) are shown. Diagnostic alleles, that is, alleles present in the rare male grandfather and not in the grandmother are shown in bold in the grandfather and in the F2 and F3 offspring. Ø indicates the presence of null alleles; 'm' indicates a rare male.

	Sample code	Apd02	Apd03	Apd05	COI
Rare male x Artemia sp. Kazakhstan	**F0 (F-Kaz 8)**	233-233	213-245	Ø-Ø	KAZSEX03
	F0 (M-Iraq 8)	233-**242**	208-**231**	115-Ø	APD02
	F2-8-2-2	233-233	**231**-245	Ø-Ø	KAZSEX03
	F2-8-2-3	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F2-8-2-4	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F2-8-2-5	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F2-8-2-6	242-**242**	**231**-245	Ø-Ø	KAZSEX03
	F2-8-2-8	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-2-3	233-233	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-2-5	233-233	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-2-10	233-233	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-2-12m	233-233	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-6-3	**242-242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-6-4	**242-242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-6-5	**242-242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-6-7m	**242-242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-8-1	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-8-2	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-8-3	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-8-4	233-**242**	**231**-245	Ø-Ø	KAZSEX03
Rare male x A. urmiana	**F0 (F-Koy 15)**	233-281	207-Ø	170-Ø	AUKOY02
	F0 (M-Iraq 15)	**254**-233	**216**-231	115-**185**	APD02
	F2-15-8-A	**254-254**	207-**216**	**185**	AUKOY02
	F315-8-A-1	**254-254**	**216**	**185**	AUKOY02
	F315-8-A-4	**254-254**	207-**216**	**185**	AUKOY02
	F315-8-A-5	**254-254**	207-**216**	**185**	AUKOY02
	F315-8-A-6	**254-254**	207-**216**	**185**	AUKOY02
	F315-8-A-7m	**254-254**	207	**185**	AUKOY02
Rare male x A. urmiana	**F0 (F-Koy 16)**	248-Ø	208-Ø	90-90	AUKOY01
	F0 (M-Iraq 16)	233-**251**	216-230	**117**-189	APD02
	F2-16-7-4	248-**251**	Ø-Ø	90-**117**	AUKOY01
	F3-16-7-4-1	248-**251**	Ø-Ø	90-**117**	AUKOY01
	F3-16-7-4-2	248-**251**	Ø-Ø	90-90	AUKOY01
	F3-16-7-4-3	248-**251**	Ø-Ø	90-**117**	AUKOY01

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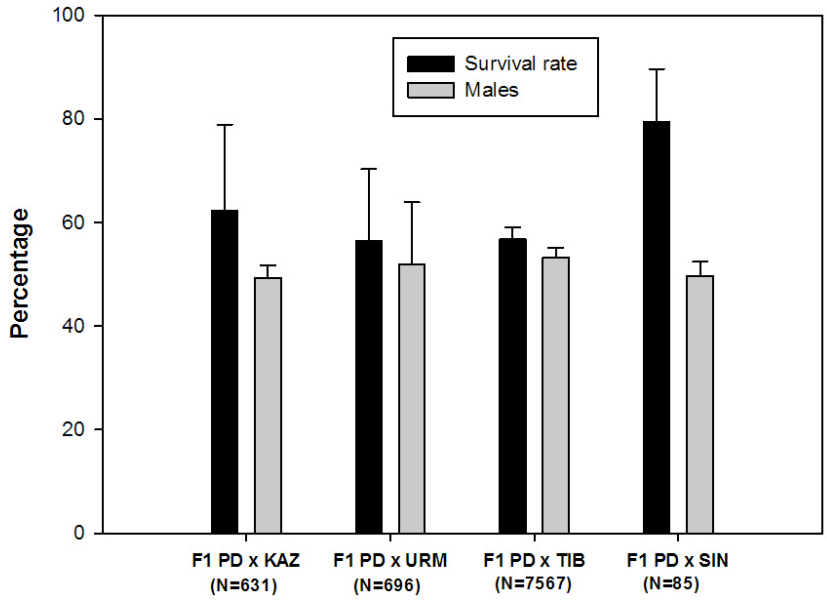
F3-16-7-4-5	248-**251**	Ø-Ø	90-**117**	AUKOY01
F3-16-7-4-7m	248-**251**	Ø-Ø	90-**117**	AUKOY01

442

442 **Figure 1. Survival rate and sex ratio (percentage of males) in the F1 hybrid offspring.** F1
443 hybrids are from parental crosses between *Artemia urmiana* (URM), *A. sinica* (SIN), *A.*
444 *tibetiana* (TIB), *Artemia* sp. from Kazakhstan (KAZ) and diploid parthenogenetic *Artemia*
445 rare males (PD). Error bars are standard deviations.
446



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449 **Figure 2. Reproductive traits (offspring quantity and quality) in F1 hybrid crosses.** The
450 viability of ovoviviparous and oviparous broods is shown. Error bars are standard deviations.
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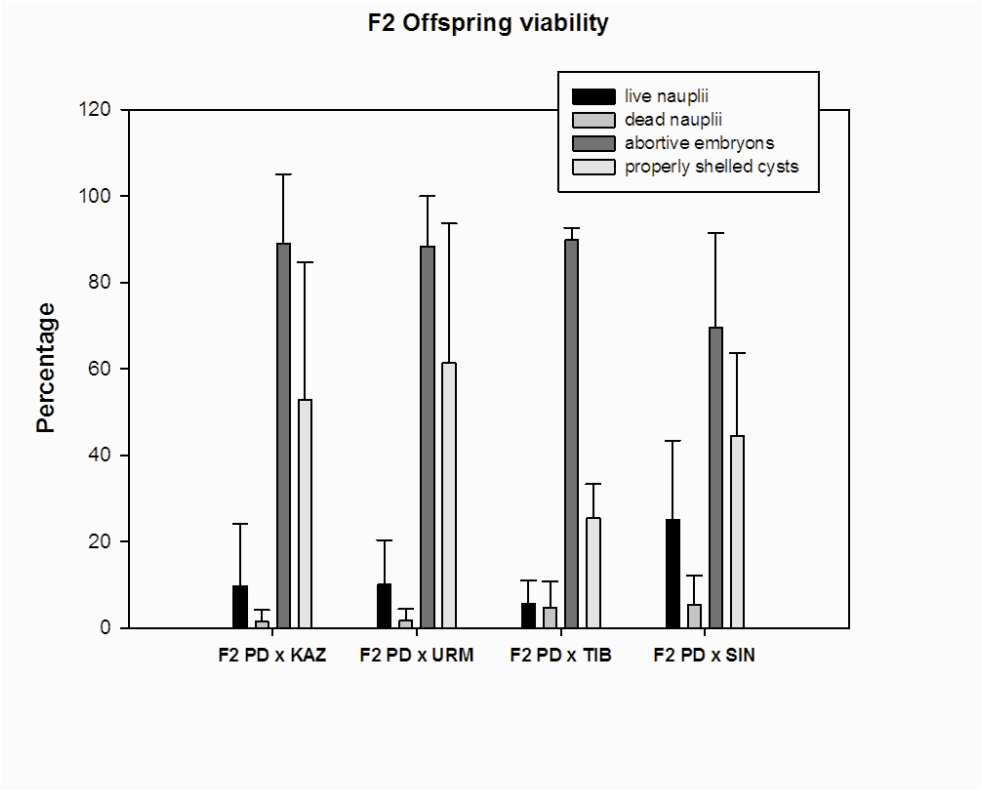

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Figure 3. Survival rate and sex ratio (percentage of males) in the F2 hybrid offspring. F2 hybrids are from crosses between F1 hybrid individuals which are obtained in the crosses between *Artemia urmiana* (URM), *A. sinica* (SIN), *A. tibetiana* (TIB), *Artemia* sp. from Kazakhstan (KAZ) and diploid parthenogenetic *Artemia* rare males (PD). Error bars are standard deviations. Asterisks ($P \leq 0.05$) indicate significant differences from 50% sex ratio (Chi-square goodness of fit test was employed).